Thrombin and Collagen Induce a Feedback Inhibitory Signaling Pathway in Platelets Involving Dissociation of the Catalytic Subunit of Protein Kinase A from an NFκB-IκB Complex

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Protein kinase A (PKA) activation by cAMP phosphorylates multiple target proteins in numerous platelet inhibitory pathways that have a very important role in maintaining circulating platelets in a resting state. Here we show that in thrombin- and collagen-stimulated platelets, PKA is activated by cAMP-independent mechanisms involving dissociation of the catalytic subunit of PKA (PKAc) from an NFκB-IκBα-PKAc complex. We demonstrate mRNA and protein expression for most of the NFκB family members in platelets. From resting platelets, PKAc was co-immunoprecipitated with IκBα, and conversely, IκBα was also co-immunoprecipitated with PKAc. This interaction was significantly reduced in thrombin- and collagen-stimulated platelets. Stimulation of platelets with thrombin- or collagen-activated IκK, at least partly by PI3 kinase-dependent pathways, leading to phosphorylation of IκBα, disruption of an IκBα-PKAc complex, and release of free, active PKAc, which phosphorylated VASP and other PKA substrates. IKK inhibitor inhibited thrombin-stimulated IκBα phosphorylation, PKAc-IκBα dissociation, and VASP phosphorylation, and potentiated integrin αIIbβ3 activation and the early phase of platelet aggregation. We conclude that thrombin and collagen not only cause platelet activation but also appear to fine-tune this response by initiating downstream NFκB-dependent PKA activation, as a novel feedback inhibitory signaling mechanism for preventing undesired platelet activation.

Platelets are small anucleate cells derived from megakaryocytes in the bone marrow, in a process in which megakaryocyte cytoplasmic extensions into microvessels are sheared from their transendothelial stems by flowing blood (1–2). Platelets play a key role in the normal homeostatic process through their ability to rapidly adhere to activated and/or injured endothelium and subendothelial matrix proteins (platelet adhesion), and to other activated platelets (platelet aggregation). Many factors bind to specific platelet receptors and regulate signaling pathways, which promote or inhibit platelet adhesion, aggregation, and secretion. In vivo, circulating platelets are continually exposed to a variety of activating factors including collagen, fibrinogen, ADP, von Willebrand Factor (vWF), thrombin, and thromboxane (3–5), as well as inhibitory factors such as endothelial-derived nitric oxide (NO), prostacyclin (PG-I2), and ADPase (3, 5–6). A strong equilibrium between the two opposing processes of platelet stimulation and inhibition is thought to be essential for normal platelet and vascular function. An impairment of this equilibrium will promote either thrombotic or bleeding disorders.

In the initial steps of platelet activation, the platelet receptor glycoproteins (GP)31b and GPVI interact with extracellular matrix (ECM) proteins, causing platelets to tether and roll on the injured endothelium or subendothelial ECM (5). Stimulation of these receptors triggers intracellular signaling cascades that activate integrin αIIbβ3 and induce the release of secondary mediators like ADP and thromboxane A2 (TXA2), leading to full platelet activation and thrombus formation. However, most of the platelets that receive stimulatory signals and initially adhere to the ECM are later detached from the ECM by blood flow and returned back into the circulation.

In human platelets, established platelet inhibitors such as NO and PG-I2 directly activate either the soluble guanylyl cyclase (sGC) or Gi-protein-coupled prostanooid membrane receptors, respectively, and thereby increase the intracellular second messengers, cGMP and cAMP, both of which have been shown to play a crucial role in platelet inhibition (6–9). The effects of the cyclic nucleotides are mediated via their respective cGMP- and cAMP-dependent protein kinases (PKG and

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PKA, which phosphorylate substrate proteins involved in platelet inhibitory pathways (6, 9).

Recently we demonstrated cross-talk between platelet stimulatory and inhibitory pathways. Activation of human platelets by vWF caused NO-independent activation of soluble guanylyl cyclase and stimulation of cGMP production and PKG, thus initiating a feedback inhibitory pathway (10). We now demonstrate that thrombin and collagen stimulation of human platelets activate another distinct feedback inhibitory mechanism based on cAMP-independent activation of PKA.

PKA is a tetrameric holoenzyme consisting of a regulatory (PKAr) subunit dimer and two catalytic (PKAc) subunits. Elevation of cAMP levels and binding of cAMP to PKAr causes dissociation of the kinase complex and release of free active catalytic subunits (11–14). However, in addition to this “classical” cAMP-dependent regulation of PKA activity, cAMP-independent activation of PKA has been demonstrated in different cell types (15–17). Some portion of PKAc molecules (independently from PKAr) is bound to IκB in an NFκB-IκB complex. Stimulation of cells with inducers of NFκB activity dissociates NFκB from IκB, leading to IκB degradation and release, and cAMP-independent activation of PKAc (15). The NFκB complex plays a significant role in megakaryocyte differentiation and maturation (18–19) and is also expressed in platelets (20), in which, however, no functional role has yet been identified.

Here we show that, in platelets, PKAc is associated with an NFκB-IκB complex, and that during platelet activation by thrombin or collagen, active PKAc is released and phosphorylates VASP\textsuperscript{Ser157} as well as other PKA substrates. This particular pathway for thrombin/collagen activation of PKA is described for the first time in platelets, and has characteristics of a novel feedback inhibitory mechanism, which would reduce the likelihood of platelet activation, particularly under weak stimulus conditions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Forskolin and Fura-2/AM were obtained from Sigma, thrombin from Roche (Mannheim, Germany), convulxin (Cvx, ligand of glycoprotein VI from the snake venom Crotalus durissus terrificus) from Axxoara (Lörrach, Germany), and collagen from Nycomed (Linz, Austria). PKC inhibitors (bisindolylmaleimide IX and I, Bis IX and I), PI3K inhibitor (wortmannin), PKA inhibitor (H-89), and IKK inhibitor VII were from Calbiochem (Darmstadt, Germany). PKB inhibitor (PKI-AKT) was from Biaffin (Kassel, Germany), 8-bromoadenosin-3’-5’-monophosphorothioate, Rp-isomer (Rp-8-Br-cAMPS) from Biolog (Bremen, Germany), and proteasome inhibitor MG-132 and Rho kinase inhibitor Y27632 from BIOMOL (Lörrach, Germany). Phospho-VASP\textsuperscript{Ser239} and phospho-VASP\textsuperscript{Ser157} antibodies were from Nanotools (Teningen, Germany). Phospho-Rap1GAP\textsuperscript{Ser27} antibodies were described previously (21). PAC-1 FITC-conjugated antibody against activated integrin \( \alpha_{IIb}\beta_{3} \), IκBα, and catalytic subunit of PKA (PKAc) were from Becton Dickinson Biosciences (Heidelberg, Germany). Phospho-p38 (Thr\textsuperscript{180}/Tyr\textsuperscript{182}) MAPK antibody was from Sigma. Antibodies against total p38, total ERK, phospho-ERK (Thr\textsuperscript{202}/Tyr\textsuperscript{204}), phospho-GSK-3 (Ser\textsuperscript{21}), phospho-IκBα (Ser\textsuperscript{22/36}), phospho-PKB (Ser\textsuperscript{473}), total PKB, PKA substrate, IκBβ, IKKα, IKKβ, and IKKγ, as well as the NFκB sampler kit, were from New England Biolabs (Frankfurt am Main, Germany). Pleckstrin antibody was from Abcam (Cambridge, UK), and MARCKS phospho-Ser\textsuperscript{159/163} antibody was from Epitomics (Hamburg, Germany). All other chemicals, reagents and solvents were of the highest purity available.

**Preparation of Washed Human Platelets**—Human platelets were prepared and used experimentally as indicated in detail below, as reported previously (10, 22) with small modifications. Blood was obtained from healthy volunteers according to our institutional guidelines and the Declaration of Helsinki, and our studies with human platelets were approved and recently (Sept. 24, 2008) reconfirmed by our local ethics committee of the University of Würzburg (Studies No. 67/92 and 114/04).

Blood was collected into ACD solution (12 mm citric acid, 15 mm sodium citrate, 25 mm d-glucose, final concentrations). Platelet-rich plasma (PRP) was obtained by 5 min of centrifugation at 330 \( \times \) g, and then aspirate (0.01 unit/ml, final concentration) was added. To reduce leukocyte contamination, PRP was diluted 1:1 with phosphate-buffered saline and centrifuged at 240 \( \times \) g for 10 min. Subsequently, the supernatant was centrifuged for 10 min at 430 \( \times \) g, then the pelleted platelets were washed once in CGS buffer (120 mm sodium chloride, 12.9 mm trisodium citrate, 30 mm d-glucose, pH 6.5), and resuspended in HEPES buffer (150 mm sodium chloride, 5 mm potassium chloride, 1 mm magnesium chloride, 10 mm d-glucose, 10 mm HEPES, pH 7.4) to a final concentration of 3 \( \times \) 10\(^8\) platelets/ml. After 15 min rest in a 37 °C water bath, washed platelets were used for experiments. Leukocyte contamination, counted using a leucocount kit (BD Biosciences), was less than 1 leukocyte per 10\(^6\) platelets.

**Aggregation Experiments**—Platelet aggregation was measured using an Apact (LabiTec) aggregometer. Washed human platelets (3 \( \times \) 10\(^8\)/ml) were preincubated with vehicle (0.01% DMSO), or 2 μM IKK inhibitor VII for 5 min. Platelet aggregation was induced by addition of different concentrations of thrombin or collagen. Aggregation in response to thrombin or collagen in the absence or presence of IKK inhibitor was registered as change in light transmission and was calculated as the area under the aggregation curve (AUC, % aggregation × s) and expressed as arbitrary units; aggregation caused by thrombin or collagen alone was designated as 100%.

**Calcium Measurement**—Calcium transients were determined with the fluorescence indicator Fura-2/AM. Platelets in PRP were loaded with Fura-2/AM for 45 min at 37 °C. Excessive dye and plasma were removed by centrifugation. The pelleted platelets were then resuspended in HEPES buffer and diluted to a cell density of 2 \( \times \) 10\(^8\) platelets/ml. Fura-2 fluorescence was measured at 340 nm with a Perkin-Elmer LS50 luminometer. Ca\(^{2+}\) (1 mm) was added immediately before the experiment.

**Immunoprecipitation and Western Blot Analysis**—For immunoprecipitation (IP) platelets were pelleted and resuspended in IP buffer (50 mm Tris, pH 7.5, 150 mm NaCl, 1 mm EDTA, 1% IGEPAL, and protease inhibitors (Complete Mini, Roche, Mannheim, Germany). Lysates were centrifuged for 20 min at 14,000 \( \times \) g and 4 °C, and supernatants were incubated with either IκBα or PKAc antibodies, or corresponding mouse or rabbit IgG as controls, overnight at 4 °C. Then protein G- or
cAMP-independent PKA Activation

### TABLE 1

PCR primers used for amplification of NFκB family cDNAs

| Protein name                        | Gene name | UniProtKB/Swiss-Prot | Forward primer | Reverse primer | Fragment size (bp) |
|-------------------------------------|-----------|----------------------|----------------|----------------|-------------------|
| Nuclear factor NFκB p105 subunit    | NFκB1     | P19838               | TGGAAAGCAGAGATGACAGAG | CGAAAGCTGCAAAACAGAAG | 544               |
| Nuclear factor NFκB p100 subunit    | NFκB2     | Q00653               | AGGCT CTGAGGAAGCCTTGT | CCGTACCAGTAGAACTCTT | 342               |
| Transcription factor p65             | REL A     | Q04206               | CGCCAGAGCAACAGAATCAC | ATCTGGAGCCTGCAAGTGT | 446               |
| Transcription factor RelB            | REL B     | Q01201               | CCACTACAGGACCTGAGAT | GTGCCGAGCATGGAAGAT | 418               |
| C-Rel proto-oncogene protein REL      | REL       | Q04864               | AAAAAATCCGGTCCGGAGAC | GCTGGAGCTTGAACCTCCTT | 473               |
| Inhibitor of NFκB kinase subunit α1  | IKKα      | 011511               | GAGAGAGGACCTGAGTGAAG | GAAGCAAAAGGCTCCCTAAT | 345               |
| Inhibitor of NFκB kinase subunit β   | IKKβ      | 014920               | TCCCGAGACTACGAGAAGCAA | GGGCAAGGGTACCAGTAC | 320               |
| NFκB inhibitor α                     | NFκB1α    | P25963               | CACACCTTGGCCCTGAAGCA | GCGCTTGCCCTACCAGTC | 466               |
| NFκB inhibitor β                     | NFκB1β    | Q15653               | ATGGACCTGAGAATGCTCTT | GACCTTCCAGCTCTCCTC | 336               |
| NFκB essential modulator             | IKK (NEMO)| Q9V689               | GGCAAAGCCCTACAAAGGCA | TGKACAGGTGACCTCAAGA | 486               |

protein A-Sepharose 4B fast flow beads (GE Healthcare, Freiburg, Germany) were added for 1 h. Immunocomplexes were washed three times with IP buffer and twice with phosphate-buffered saline, then precipitated proteins were detected by immunoblotting.

For Western blotting, total platelet and HL-60 cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and the membranes incubated with appropriate primary antibodies overnight at 4 °C. For visualization of the signal, goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase was used as secondary antibodies, followed by ECL detection (GE Healthcare). Blots were analyzed densitometrically using NIH Image J software for uncalibrated optical density.

RNA Isolation from Platelets, and RT-PCR Analysis—Total RNA was extracted from washed platelets (1 × 10^9) and HL-60 cells (1 × 10^7) cells by TRIzol Reagent (Invitrogen, Karlsruhe, Germany). Isolated RNA was reverse transcribed using the first strand cDNA synthesis kit (Stratagene, Waldbronn, Germany) according to the manufacturer’s instructions. 1 μl of cDNA synthesis reaction was subjected to PCR and amplified for 35 cycles. Primers sequences and expected sizes of transcribed gene products are given in Table 1. The purity of platelet cDNA was controlled with primers specific for genomic DNA; only those samples negative for genomic DNA contamination were selected for PCR amplification. The specificity of all PCR products was confirmed by sequencing using product-specific PCR primers.

Flow Cytometry—The level of integrin αIIbβ3 activation was determined using PAC-1 FITC-conjugated antibody against activated integrin αIIbβ3. PAC-1 FITC-conjugated antibody (2 μl) was directly added to 20 μl of platelets (3 × 10^9/ml), diluted with 400 μl of phosphate-buffered saline/0.3% bovine serum albumin, and subsequently analyzed in a Becton Dickinson FACS Calibur using CELLQuest software, version 3.1f. The platelet population was identified by its forward and side scatter distribution, and 10,000 events were analyzed for mean fluorescence.

cAMP and cGMP Measurements—Levels of cAMP and cGMP were evaluated using a cAMP EIA kit and cGMP EIA kit, respectively, following the manufacturer’s instructions (Cayman Chemical, Hamburg, Germany).

NFκB Activity Assay—NFκB activity from platelets and HL-60 cells was assessed by the binding of activated p65 to a consensus binding site on biotinylated oligonucleotides, measured by an ELISA kit from Panomics (Fremont, CA) according to the manufacturer’s instructions and was expressed as arbitrary units of DNA binding.

Data Analysis—All experiments were performed at least in triplicate, and data shown are means ± S.E. The n-values refer to the number of experiments, each made with different blood donors. Differences between groups were analyzed by Student’s t test. ANOVA was used for time and dose-response experiments. p < 0.05 was considered statistically significant.

RESULTS

Cyclic Nucleotide-independent Phosphorylation of VASP_{Ser157} and Other PKA Substrates in Thrombin- and Collagen-stimulated Platelets—VASP is one of the major PKA and PKG substrates in platelets and has been used to monitor the activation state of both kinases in numerous studies (23). Treatment of platelets with thrombin resulted in phosphorylation of VASP at Ser^{157}, the site preferred by PKA (Fig. 1A). VASP Ser^{157}, the site more preferred by PKG, was not phosphorylated after thrombin treatment (Fig. 1A). To investigate whether thrombin-induced VASP Ser^{157} phosphorylation was related to elevated cyclic nucleotide levels, we determined cAMP and cGMP concentrations. Increase of cGMP in thrombin- and collagen-stimulated platelets has been described in several publications (reviewed in Ref. 24), whereas cAMP has been shown to decrease in thrombin-stimulated platelets (25–26). In our experiments, we could not detect any increase of cGMP in thrombin- or collagen-stimulated platelets (Fig. 1B), which directly correlated with the unchanged, or even slightly reduced, VASP Ser^{157} phosphorylation observed in response to thrombin in Fig. 1A. We detected a significant decrease in cAMP only in thrombin-stimulated, not collagen-stimulated platelets. cAMP concentration in control was 17.1 ± 0.6 pmol/10^9 platelets, in collagen-stimulated platelets 16.5 ± 0.7 pmol/10^9 platelets, and in thrombin-stimulated platelets 11.4 ± 0.5 pmol/10^9 platelets (n = 4, p < 0.05). cGMP content in control was 0.11 ± 0.02 pmol/10^9 platelets and was not significantly changed in thrombin- (0.12 ± 0.03), or collagen- (0.13 ± 0.03) stimulated platelets (Fig. 1B). In contrast, forskolin and sodium nitroprusside (SNP) (5 μM each) elicited expected cAMP and cGMP increases, respectively.

To investigate whether thrombin-induced VASP phosphorylation at Ser^{157} was mediated by PKA, we used two different PKA inhibitors, H-89 that inhibits ATP binding to PKAc and Rp-8-Br-cAMPS that is a competitive inhibitor of cAMP-binding sites in PKAr. Both inhibitors strongly decreased the VASP phosphorylation caused by forskolin, an agent which activates...
adenylyl cyclase, and as a consequence, cAMP-dependent PKA stimulation. In contrast, thrombin (Fig. 1A) (and collagen, data not shown) stimulated VASP phosphorylation was only inhibited by H-89, not by Rp-8-Br-cAMPS, suggesting that thrombin action was cAMP-independent. Thrombin or collagen stimulation of VASP\textsuperscript{Ser157} phosphorylation was enhanced by a very low forskolin concentration (100 nM), suggesting that the cAMP-dependent (forskolin) and cAMP-independent effects were additive (see supplemental Fig. S1). We determined that VASP is not the only VASP substrate phosphorylated in response to thrombin and collagen stimulation of platelets by examining the phosphorylation of Rap1GAP2, which has been recently identified as a substrate of PKA and PKG in platelets (21, 27). In our experiments, Rap1GAP2 was indeed phosphorylated in thrombin- and collagen-stimulated platelets (Fig. 1C). To gain a broader view of PKA-mediated phosphorylation events, we used a PKA substrate antibody that recognizes the RR(S/T) consensus phosphorylation site when S or T is phosphorylated. Numerous phosphoproteins are recognized by this antibody after forskolin, as well as thrombin or collagen treatment. Several of them (arrows in supplemental Fig. S2) have the same molecular weight, suggesting that thrombin and collagen activate pathways that lead to phosphorylation of PKA substrates, which are also targeted by forskolin. Taken together, our data (Fig. 1, A–C) suggest that, unlike forskolin, thrombin and collagen induce phosphorylation of VASP and other PKA substrates independently of cyclic nucleotides. Next, we evaluated the concentration and time dependence of VASP\textsuperscript{Ser157} phosphorylation in thrombin-, collagen-, and forskolin-stimulated platelets (Fig. 2). Platelets were stimulated with thrombin (0.01, 0.05, 0.1 unit/ml), collagen (1, 5, 10 μg/ml), or forskolin (0.1, 0.5, 1 μM) for 2 min (Fig. 2A), or with 0.01 unit/ml of thrombin, 1 μg/ml of collagen, and 0.1 μM forskolin from 1 to 10 min (Fig. 2B). VASP\textsuperscript{Ser157} phosphorylation was independent of collagen and thrombin concentration, but dependent on forskolin concentration (Fig. 2A). VASP\textsuperscript{Ser157} phosphorylation induced by all three compounds was not significantly changed from 1 to 10 min of stimulation (Fig. 2B).

Analysis of Intracellular Mechanisms of Thrombin- and Collagen-induced VASP\textsuperscript{Ser157} Phosphorylation in Platelets—To exclude that other S/T kinases, which are also activated by thrombin or collagen, might be involved in VASP\textsuperscript{Ser157} phosphorylation, we analyzed effects of different kinase inhibitors using established kinase substrates as controls. VASP\textsuperscript{Ser157} has been shown to be directly phosphorylated \textit{in vitro} by protein kinase C (PKC) (28). In thrombin-stimulated platelets VASP\textsuperscript{Ser157} has been described to be phosphorylated by PKC-dependent and -independent mechanisms (29–30). To investigate whether PKC phosphorylates VASP\textsuperscript{Ser157} in isolated platelets (SNP) (both 5 μM, 2 min)-stimulated platelets were used as positive controls. cAMP/cGMP concentrations were measured as described under “Experimental Procedures.” cAMP concentration significantly decreased only in thrombin-stimulated platelets. Results are means \pm S.E., n = 4, + p < 0.05 compared with control. A, platelets were untreated or preincubated with PKA inhibitors (H-89, 10 μM; Rp-8-Br-cAMPS, 200 μM), prior to stimulation with thrombin or forskolin. Platelet activation was monitored by p38 phosphorylation. Immunoblots were scanned and quantified by the Image J program, the intensity of the VASP\textsuperscript{Ser157} signal normalized to the total p38 signal, and then this ratio for each sample was expressed relative to the ratio for thrombin stimulation which was designated as 1. H-89 inhibited both thrombin- and forskolin-stimulated VASP\textsuperscript{Ser157} phosphorylation, whereas Rp-8-Br-cAMPS inhibited only forskolin-stimulated VASP\textsuperscript{Ser157} phosphorylation. Results are means \pm S.E., n = 4, + p < 0.05 compared with the control; *p < 0.05 compared with the corresponding stimulus. B, cAMP and cGMP levels in thrombin- and collagen-stimulated platelets. Forskolin- and sodium nitroprusside (SNP) (both 5 μM, 2 min)-stimulated platelets were used as positive controls. cAMP/cGMP concentrations were measured as described under “Experimental Procedures.” cAMP concentration significantly decreased only in thrombin-stimulated platelets. Results are means \pm S.E., n = 4, + p < 0.05 compared with control. C, platelets were incubated with thrombin (0.01 unit/ml) or collagen (10 μg/ml), or each of these after preincubation with H89 (10 μM, 10 min) as indicated, and analyzed for VASP\textsuperscript{Ser157} and Rap1GAP2\textsuperscript{Ser7} phosphorylation, or total Rap1GAP2 expression. Results are representative of three independent experiments.

![Figure 1](image-url)
PKB-PI3K- (which is upstream of PKB), and PKA-dependent phosphorylation of VASP and GSK3, we used the PI3K inhibitor wortmannin, the PKB inhibitor PKI-AKT, and the PKA inhibitor H-89 (Fig. 3, B–D). The PI3K inhibitor wortmannin completely inhibited thrombin-induced GSK3 phosphorylation, partly inhibited thrombin-induced VASP Ser157 phosphorylation and had no effect on forskolin-induced phosphorylation of either protein, indicating that thrombin-induced VASP Ser157 phosphorylation is only partly mediated by PI3K-dependent pathways (Fig. 3B). However, the PKB inhibitor (PKI-AKT) inhibited only GSK3 phosphorylation, not VASP Ser157 phosphorylation, and the PKA inhibitor, H-89, dose-dependently inhibited thrombin-induced phosphorylation of VASP Ser157, GSK3, and PKB (Fig. 3, C and D). All three inhibitors strongly reduced thrombin-induced integrin αIIbβ3 activation (Fig. 3C and data not shown). These data indicate that (i) PI3K, but not PKB, is upstream of thrombin-induced VASP Ser157 phosphorylation, and suggest (ii) that the commonly used PKA inhibitor, H-89, also strongly inhibits the PI3K/PKB pathway in platelets.

In endothelial cells, thrombin-induced VASP Ser157 phosphorylation has also been shown to be dependent on RhoA kinase and MEKK/ERK pathways (16). Here we tested whether any of these kinases might be involved in thrombin-induced VASP Ser157 phosphorylation in platelets (Fig. 3, E and F). In thrombin- and collagen-stimulated platelets, the Rho kinase inhibitor Y27632 specifically inhibited only phosphorylation of its established substrate myosin light chain 2 (MLC) but had no effect on VASP Ser157 and p38 MAP kinase phosphorylation (Fig. 3E). Even high concentrations (up to 50 μM) of U0126, an inhibitor of the MEKK/ERK pathway, specifically inhibited only ERK phosphorylation, not VASP Ser157 phosphorylation, in collagen (data not shown)- or thrombin (Fig. 3F)-stimulated platelets. We conclude that thrombin- and collagen-induced phosphorylation of VASP Ser157 most likely involves PI3K and PKA, but none of the other kinases are suggested to phosphorylate VASP at this site.

Expression of NFκB Family Proteins and mRNA in Human Platelets—The NFκB transcription factors are members of three families (IKK, NFκB, and IκB) of proteins (33). Expression of only some of the NFκB family members has been demonstrated in platelets (20). Therefore we performed a more comprehensive examination of the protein and mRNA expression of the most common members of the NFκB, IκB, and IKK family in platelets, using HL-60 cells as a nucleated positive control (Fig. 4). The purity of platelet cDNAs was controlled using primers specific for genomic DNA (data not shown). All three IKK (α, β, and γ) family members are expressed in platelets and HL-60 cells, with IκBβ protein being even more strongly expressed in platelets than in HL-60 cells. Of the NFκB/Rel family, Rel B protein was found to be most strongly expressed, and C-Rel undetectable, in platelets (Fig. 4). Of the IκB family, Bcl-3 has been described as expressed in platelets (34), and we found that IκBα and IκBβ proteins are also expressed in platelets, however at lower levels than in HL-60 cells (Fig. 4).
FIGURE 3. VASP<sup>Ser157</sup> phosphorylation in thrombin- and convulxin-stimulated platelets is not mediated by PKC, PKB, RhoA kinase, or MEKK/ERK pathways, but may partially involve PI3K. Washed human platelets (3 × 10<sup>8</sup>/ml) were incubated for 1 min with thrombin (0.01 unit/ml), convulxin (5 ng/ml), or forskolin (0.1 μM) as indicated, or each of these after preincubation for 10 min with either (A) PKC inhibitors bisindolylmaleimide I or I<sub>X</sub> (BisI, BisIX, both 5 μM), (B) wortmannin (0.1–0.5 μM), (C) PKA inhibitor (H-89, 10 μM), or PKB inhibitor (PKI-AKT, 10 μM), (D) PKA inhibitor (H89, 5–20 μM), (E) Rho kinase inhibitor (Y27632, 10 μM), or (F) MEKK1 inhibitor (U0126, 10–50 μM). Results were analyzed by flow cytometry for integrin αIIbβ3 activation (PAC-1 binding) and by Western blotting for VASP, MARCKS, pleckstrin, GSK3, PKB, p38, MLC, and ERK phosphorylation. PAC-1 binding (A, C) represents integrin αIIbβ3 activation and is expressed as % of the thrombin effect, which was designated as 100%. Immunoblots (D) were scanned and the intensity of bands quantified by the Image J program. VASP<sup>Ser157</sup> and PKB<sup>Ser173</sup> phosphorylation were normalized to the total PKB signal. Results are means ± S.E., n = 4. A and C, * significant difference from control; *, significant difference from thrombin- or convulxin-stimulated platelets, respectively. D, ** significant difference from thrombin- or forskolin-stimulated platelets, respectively.
cAMP-independent PKA Activation in Thrombin- and Collagen-stimulated Human Platelets Is Mediated by Dissociation of PKAc from an NFκB-IκB Complex—After the first description that PKA activity may be regulated independently of cAMP by dissociation of PKAc from an NFκB-IκB complex (15), this mechanism was described in several cell types (16–17). In nucleated cells, activation of PKA is associated with increased transcriptional activity of NFκB. However, the function of NFκB complexes in anucleate platelets has not been described. We therefore examined whether (i) PKAc is also associated with the NFκB-IκB complex in platelets, (ii) PKAc is released from this complex in agonist-stimulated platelets, (iii) NFκB is activated in agonist-stimulated platelets, and (iv) whether cAMP-independent PKA activation in thrombin-stimulated platelets has functional relevance.

In resting platelets, PKAc was co-immunoprecipitated with IκBa (Fig. 5, A and B), and IκBa was co-immunoprecipitated with PKAc (Fig. 5, C and D). In thrombin- and collagen-stimulated platelets, both the amount of PKAc co-precipitated with IκBa, and the amount of IκBa co-precipitated with PKAc was significantly reduced (Fig. 5, E and F). This reduced binding of PKAc to IκBa indicates that in platelets, as in other cell types, activation disrupts the IκBa-PKAc complex, resulting in the release of free active PKAc. Disruption of the IκBa-PKAc complex and phosphorylation of VASP Ser157 in thrombin-stimulated platelets was inhibited by preincubation with IKK inhibi-
tor (Fig. 5G). Next, we calculated the amount of PKAc that forms a complex with IκBα. For this, we immunoprecipitated IκBα and performed a Western blot analysis of PKAc in the precipitate, as well as in defined amounts of platelet lysate and purified PKAc (Fig. 5G). Immunoblots were scanned and the intensity of the PKAc bands was quantified using the Image J software. Regression analysis of PKAc band intensity showed a linear correlation with μg of loaded platelet proteins and ng of purified PKAc. From these data we observed that 1 μg of platelet protein contains 0.58 ± 0.03 ng of PKAc, in agreement with our previous calculations (35). Collectively, the data in Fig. 5G can be used to deduce that 1.63% of total platelet PKAc forms a complex with and is co-precipitated with IκBα. However, this amount of PKAc may be underestimated because it depends on the efficiency with which the IκBα antibody immunoprecipitates the IκBα-PKAc complex.

**NFκB Activation in Thrombin-stimulated Platelets**—In nucleated cells, activation of NFκB leads to phosphorylation, ubiquitination, and degradation of IκB proteins. In thrombin-stimulated platelets, IκBα was phosphorylated at Ser32/36, and the phosphorylation was blocked by IKK inhibitor (panel + IKK inh in Fig. 6A). Also, the level of total IκBα was significantly decreased after 10 min of thrombin stimulation (bar graph). This degradation of IκBα was prevented by preincubation with the proteasome inhibitor MG-132 (panel + MG-132 in Fig. 6A). Activated NFκB binds to its DNA consensus sequence, and NFκB activity can be measured by ELISA with biotinylated oligonucleotides containing this consensus sequence. In both platelets and HL-60 cells (including as a positive control), thrombin activated NFκB and induced binding of activated NFκB to target oligonucleotides (Fig. 6B). NFκB DNA binding increased in thrombin-stimulated platelet lysate continually over the 1-h incubation period, whereas it peaked after 10 min in HL60 cells.

**Inhibition of IKK Potentiates Thrombin- and Collagen-stimulated Platelet Activation without Significant Changes in Calcium Mobilization**—To demonstrate the physiological relevance of thrombin- and collagen-induced VASPSer157 phosphorylation, we investigated effects of IKK inhibitor on integrin αIIbβ3 activation, calcium mobilization, and platelet aggregation. Preincubation of platelets with different concentrations of IKK inhibitor strongly reduced thrombin-induced VASP Ser157 phosphorylation, but had no effect on forskolin-induced VASP Ser157 phosphorylation (Fig. 6C). IKK inhibitor, at low concentrations up to 5 μM, not only inhibited VASP phosphorylation, but also significantly increased thrombin-stimulated integrin αIIbβ3 activation (PAC-1 binding). In collagen-stimulated platelets IKK inhibitor also reduced VASP Ser157 phosphorylation, however the potentiating effect on PAC-1 binding was very low and not significant (data not shown). Preincubation of platelets with IKK inhibitor caused only slight (not significant) potentiation of thrombin-, but not collagen-induced calcium mobilization (supplemental Fig. S3), indicating that changes other than in intracellular calcium are involved in NFκB effects on platelets. The effects of all concentrations of thrombin (0.001, 0.01, 0.02, and 0.05 unit/ml) and collagen (1, 5, and 10 μg/ml) on the early phase of platelet aggregation were significantly potentiated by IKK inhibitor VII
**cAMP-independent PKA Activation**

![Figure 7](image)

**FIGURE 7.** IKK inhibitor potentiates thrombin- and collagen-stimulated platelet aggregation. Washed human platelets (3 × 10⁶/ml) were preincubated with vehicle (0.01% DMSO) or 2 μM IKK inhibitor VII for 5 min. Platelet aggregation was stimulated by addition of 0.01 unit/ml thrombin or 10 μg/ml collagen. A and B, representative aggregation traces showing potentiating effect of IKK inhibitor VII on thrombin (A)- and collagen (B)-induced platelet aggregation. The aggregation in response to thrombin and thrombin + IKK inhibitor VII (C), as well as collagen and collagen + IKK inhibitor VII (D) were calculated as the area under the aggregation curve (AUC, % aggregation × s) and expressed as arbitrary units; aggregation caused by thrombin or collagen alone was designated as 100%. Results are means ± S.E., n = 6, +, significantly different from control in C and D.

(Fig. 7, and other data not shown, for several thrombin and collagen concentrations).

**DISCUSSION**

Platelet inhibitory mechanisms play an important role in preventing circulating platelets from undesired activation. It is widely accepted that cyclic nucleotides (cAMP and cGMP) and their corresponding protein kinases (PKA and PKG) are key players in regulation of platelet inhibition. However, recently, several other mechanisms involved in platelet inhibition have been described. For example, constitutive activity of a novel platelet receptor G66-B is involved in inhibition of GPVI and CLEC-2 signaling, and speculated to have an important physiological role in helping to prevent platelet activation in vivo (36). The canonical Wnt signaling was recently described in platelets and shown to be involved in inhibition of platelet adhesion, shape change, dense granule secretion, RhoA activation, and aggregation (37). Activation of peroxisome proliferator-activated receptor γ (PPAR-γ) inhibited collagen-stimulated platelet aggregation, intracellular calcium mobilization, P-selectin exposure, and thrombus formation (38). Protease nexin-1 (PN-1), stored in α-granules and released during platelet activation, is involved in inhibition of tissue factor-induced thrombin generation, low-dose thrombin-induced P-selectin surface expression, and platelet aggregation (39). An adapter protein disabled-2 (DAB2), which is also associated with α-granules and secreted from agonist-stimulated platelets, inhibited platelet integrin αIIbβ3 activation and platelet aggregation by binding to the extracellular region of αIIb integrin (40). Diadenosine 5′, 5″-P²,P⁴-tetraphosphate (Ap₄A), a component of platelet dense granules, is involved in inhibition of ADP-induced platelet activation (41). Interestingly, the last three inhibitors of platelets (PN-1, DAB2, and Ap₄A) act as feedback inhibitors that are activated only in agonist-stimulated platelets.

Here we describe a novel platelet feedback inhibitory mechanism involving PKA, which is activated in thrombin- and collagen-stimulated platelets. In our studies, thrombin and collagen activation of platelets increased phosphorylation of the VASP protein, which is a major substrate of PKA, a well-known inhibitor of platelet signaling. PKA is normally activated by cAMP-dependent dissociation of the regulatory and catalytic holoenzyme PKA-R-PKAc. However, platelet activation by soluble agonists commonly involves a reduction in intracellular cAMP levels. For example, ADP and epinephrine induce Gₛ-protein-dependent inhibition of adenylyl cyclases. Thrombin stimulation of platelets has been reported to decrease cAMP by PKB- and PKC-dependent phosphorylation and activation of PDE3A (25–26). Our experiments also confirmed that thrombin decreases cAMP levels in platelets (Fig. 1B). Furthermore, we could show that thrombin activation of PKAc in platelets is cAMP independent, instead involves dissociation of an NFkB-IκBα-PKAc complex to release free PKAc (Fig. 5), which in turn opposes the platelet activation initiated by thrombin.

Although PKA is mainly activated by cAMP, a fraction of total cellular PKA forms a complex with NFkB-IκBα proteins and may be released upon NFkB activation by different stimuli (15–16), including thrombin in endothelial cells (15–16). Expression of NFkBα (p65) and IκBα proteins, and their activation by thrombin and other agonists have been shown in platelets (20). During preparation of this report for publication, three reports concerning NFkB expression and function in platelets were published (42–44). In contrast to our data, authors of these works report that NFkB plays a significant role in platelet activation. In the report of Malaver et al. (42), two NFkB inhibitors (BAY 11-783 and Ro 106-9920) were used at high concentrations (up to 50 μM) without any appropriate controls for their specificity. In our study, we also used both of these inhibitors and found that even much lower concentrations (less than 5 μM), independently of NFkB, significantly inhibited several platelet activation pathways including PI3K/PKB, as well as inhibiting integrin αIIbβ3 activation, aggregation, and P-selectin expression (data not shown). The conclusions of this report (42) were also questioned by an editorial (45) published in the same issue of the Journal of Thrombosis and Hemostasis. An article by Lee et al. (43) reported that 5–20 μM of the same NFkB inhibitor (BAY 11-783) had significant inhibitory effects, in rat platelets. Species-dependent differences of NFkB expression and function may exist in platelets, because our studies found that, in contrast to human platelets, thrombin- and collagen-stimulated mouse platelets did not show any significant VASP phosphorylation (data not shown), indicating that at least this NFkB function is undetectable in mouse platelets. In agreement with our data (Fig. 4), Spinelli et al. (44) demonstrated expression of most NFkB family members in platelets; however, in contrast to the two previous works (42–43), reported significant platelet inhibitory effects already at low (0.5–5 μM) concentrations of BAY 11–783 (44). However,
to prove the specificity of BAY 11-783, the authors used a rather questionable method of introducing recombinant full-length proteins into platelets.

The classical role of NFκB is associated with the regulation of gene expression, a function which can be excluded in anucleate platelets. Here we describe a new function of NFκB proteins involving CAMP-independent activation of PKA in thrombin- and collagen-stimulated platelets, which we propose represents a novel platelet inhibitory feedback mechanism.

The first indication of PKA activation in thrombin- and collagen-stimulated platelets was phosphorylation of the PKA substrate VASP Ser157. Furthermore, inhibition of PKAr by Rp-8-pCPT-cAMPS had no effect on thrombin-induced VASP Ser157 phosphorylation (Fig. 1A), indicating that under these conditions PKA was activated by a CAMP-independent pathway. The alternative, that PKA substrates could be phosphorylated by other kinases activated by thrombin and collagen, was examined.

Because several studies described PKC as a kinase which phosphorylates VASP Ser157 (28–30), we analyzed this possibility in detail, using more than 10 different PKC inhibitors. We verified the functional activity of each inhibitor by measuring its effect on the phosphorylation of established PKC substrates (MARCKS, pleckstrin) and on the inhibition of integrin αIIbβ3 activation. Most PKC inhibitors inhibited PKC activity (assessed by MARCKS and pleckstrin phosphorylation and integrin αIIbβ3 activation) with different efficacy; however, none of them inhibited thrombin- or collagen-induced VASP Ser157 phosphorylation in platelets (Fig. 3A and data not shown). However, direct activation of PKC using PMA did induce VASP Ser157 phosphorylation, which was inhibited by H-89 (data not shown). PMA is a well-known inducer of NFκB activation in platelets, in a fashion similar to that of thrombin or collagen (20). Based on these data, we conclude that PMA-induced VASP Ser157 phosphorylation in platelets is not mediated by PKC itself, but rather by PKC-dependent NFκB activation followed by the release of active PKAc from an IκBα-PKAc complex.

In our experiments, thrombin-induced VASP Ser157 phosphorylation was partially blocked by the P13 kinase inhibitor wortmannin (Fig. 3B), which can also inhibit PKB activity downstream of P13K. However, the wortmannin effect was not mediated by PKB, because the PKB inhibitor PKI-AKT (which inhibits phosphorylation of the PKB substrate GSK3) had no effect on VASP Ser157 phosphorylation (Fig. 3C). A second important observation from these experiments was the lack of specificity of the commonly used PKA inhibitor H-89. We expected that inhibition of PKA by H-89 would potentiate platelet activation, but in all of our experiments, H-89 dose-dependently and significantly inhibited thrombin- or collagen-induced integrin αIIbβ3 activation, which can be explained by the inhibition of P13 kinase/PKB pathways (inhibition of P-GSK3, Fig. 3C, and P-PKB, Fig. 3D). Unfortunately, in platelets only relatively high concentrations (greater than 5 μM) of H-89 can be used, because lower concentrations had no effect on PKA activity assessed by VASP phosphorylation (Fig. 3D and data not shown). We also tested other commercially available PKA inhibitors (KT5720 and PKI) at different concentrations. KT5720 required even higher (greater than 20 μM) concentrations to inhibit VASP phosphorylation and at these concentrations KT5720 started to inhibit other kinases (P13K, PKB, PKC) and integrin αIIbβ3 activation (data not shown). PKI (5 μM), which was previously shown by us to have no effect on platelet PKA activity and higher concentrations even strongly stimulated platelet aggregation without inhibition of PKA (22).

In endothelial cells, thrombin-induced VASP Ser157 phosphorylation is mediated by RhoA/Rho kinase and MEKK1 that are downstream of the Gα13 pathway (16). In platelets, thrombin also stimulates the Gα13 pathway (46) and its downstream effectors MEKK1 and Rho kinase, however, in contrast to endothelial cells, neither of these kinases is involved in thrombin-induced VASP Ser157 phosphorylation (Fig. 3, E and F).

Thrombin-induced NFκB activation in both platelets and HL-60 cells (used as a positive control) was demonstrated by IκBα phosphorylation and degradation, and by increased DNA-binding of activated NFκB. We could show that in platelets, like in other cell types (15, 17, 47), a portion of PKAc binds to IκBα and co-immunoprecipitates with IκBα. This interaction was significantly reduced in thrombin- and collagen-stimulated platelets, indicating release of catalytically active PKAc from IκBα (Fig. 5). In addition, low dose IKK inhibitor (up to 5 μM) inhibited thrombin-induced disruption of the IκBα-PKAc complex (Fig. 5G), inhibited thrombin-induced VASP Ser157 phosphorylation, and potentiated integrin αIIbβ3 activation, without having any effect on forskolin- and thus CAMP-dependent VASP Ser157 phosphorylation (Fig. 6C). Importantly, inhibition of NFκB function by IKK inhibitor VII significantly potentiated thrombin- and collagen-stimulated platelet aggregation, especially the early phase (Fig. 7).

Platelet integrin activation and aggregation are consequences of several, partially independent, mechanisms, which include protein phosphorylation/dephosphorylation, calcium mobilization, changes in protein/protein interactions, etc. The potentiating effect of IKK inhibitor on the final steps of platelet activation (integrin activation, aggregation) is small, not more than 20%, and is potentially a composite of effects on one or more of the above-mentioned mechanisms, although not calcium mobilization (which was not significantly influenced by IKK inhibitor, see supplemental Fig. S3). A thorough analysis of intracellular mechanisms involved in mediation of NFκB functions in platelets merits future investigation.

Based on our data, we propose the following mechanisms and effects of activation of an NFκB-IκBα-PKAc complex in platelets (Fig. 8). Activation of thrombin and collagen receptors stimulates P13 kinase/PKB/PKC pathways (for the sake of simplicity, other well-established signaling cascades are omitted from the scheme), ultimately leading to platelet activation. Both receptors partly utilize the P13 kinase (and possibly other yet to be established) pathways to activate the NFκB complex, which leads to degradation of IκB, release of active PKAc, and consequently to the phosphorylation of VASP and other PKA substrates involved in platelet inhibitory pathways. However, our experiments do not completely rule out the possibility that other, yet unidentified kinases besides PKA can phosphorylate

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S. Gambaryan, unpublished data.
In summary, we show here that most of the NFκB family members are expressed in platelets at the mRNA and protein level. Stimulation of platelets with thrombin or collagen disrupts an NFκB-IXaBα-PKAc complex, which leads to PKA activation and phosphorylation of VASP and other PKA substrates involved in platelet inhibitory pathways. An IKK inhibitor inhibited this chain of events and potentiated αIIbβ3 integrin activation and aggregation of platelets, suggesting that NFκB-dependent PKA activation represents a novel feedback inhibitory mechanism to modulate platelet functions.

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