DUAL REGULATION OF PLATELET PROTEIN KINASE B

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Summary

Protein kinase B (PKB) is a serine/threonine kinase which is activated by growth hormones and implicated in prevention of apoptosis, glycogen metabolism and glucose uptake. A key enzyme in PKB activation is phosphatidylinositide 3-kinase (PI-3K), which triggers the dual phosphorylation of PKB by phosphatidylinositol-dependent-kinases (PDKs). Here we report, that the major PKB subtype in platelets is PKBα, which is activated by phosphorylation of Thr308 and Ser473, and has a constitutively phosphorylated Tr450 that does not contribute to PKB activation. α-Thrombin and thrombopoietin (TPO) activate PKBα via PI-3K and trigger the concurrent phosphorylation of Thr308 (via PDK1) and Ser473 (via a not yet identified PDK2). In addition, α-thrombin activates a PI-3K independent pathway involving phospholipase Cβ and calcium-dependent protein kinase C subtypes (PKCα/β). This route is specific for phosphorylation of Ser473 and can be initiated by direct PKC activation with phorbol ester or purified active PKC catalytic fragment in platelet lysate. Different degrees of Ser473 and Thr308 phosphorylation correlate with different degrees of enzyme activity. These data reveal a PI-3K independent PKB activation in which PKCα/β regulates the phosphorylation of Ser473 in PKBα. The independent control of the two phosphorylation sites may contribute to fine-regulation of PKBα activity.
Introduction

Protein kinase B (PKB, also known as RAC or Akt kinase) is a 57 kDa, phospholipid-dependent serine/threonine kinase and a product of the oncogene v-Akt of the acutely transforming retrovirus Akt8 (1). It was first isolated from a rodent T-cell lymphoma and had the capacity to induce cell transformation (2). Subsequently, PKB was reported to prevent apoptosis (3), to regulate glyconeogenesis by phosphorylation of glycogen synthase kinase 3 (GSK3) (4) and to control glucose uptake by inducing translocation of the glucose transporter GLUT-4 to the plasmamembrane (5). In addition, PKB modulates protein synthesis by activation of p70 ribosomal S6 kinase (6).

The human genome encodes for at least three different PKB genes, which display more than 80% sequence homology and are named PKBα, -β and -γ. They are differentially distributed with each tissue containing at least one PKB subtype (2). PKB is activated in response to agonists such as platelet-derived growth factor, epidermal growth factor, insulin, nerve growth factor and α-thrombin. Activation takes less than a few minutes and is prevented by inhibitors of phosphatidylinositol 3-kinase (PI-3K) (5,6,7), indicating that this enzyme is an upstream regulator of PKB. The PI-3K products PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ function to tag cytosolic, inactive PKB together with phosphatidylinositol-dependent-kinases 1 and 2 (PDK1/2) to the plasmamembrane, thereby initiating PKB activation. Binding of PKB to PtdIns(3,4)P₂, PtdIns(3,4,5)P₃ and PDK1/2 induces a conformational change and a slight increase in PKB activity (8,9,10). Membrane attachment is a prerequisite for phosphorylation by PDK1/2, which leads to complete PKB activation and protects active PKB against dephosphorylation and inactivation by cytosolic phosphatases. In addition to the PI-3K-mediated activation of PKB, PI-3K-independent activation has been reported. Examples are osmotic or thermic stress, treatments that raise cAMP, or addition of okadaic acid (11,12), an inhibitor of the Ser/Thr phosphatase PP-1 and PP-2.

PKB consists of an N-terminally located PH-domain linked to a centrally located catalytic domain, which is connected to a short C-terminal tail. The catalytic domain displays high homology to the catalytic domains of cAMP-dependent protein kinase A (PKA; 65 %) and protein kinase C (PKC; 75 %) and contains a Thr308 phosphorylation site. A second phosphorylation site is Ser473, which is located in the C-terminal tail (13). The PKB subtypes differ with respect to these phosphorylation sites, which are Thr308/Ser473 in PKBα and Thr309/Ser474 in PKBβ. Due to a C-terminal truncation, PKBγ has only one phosphorylation site (Thr305) (14). Substitution of Thr308 or Ser473 for Asp by site-directed mutagenesis abolishes the activation of PKBα, suggesting that phosphorylation of both residues...
is required for full enzyme activity (13). The PtdIns(3,4)P₂/PtdIns(3,4,5)P₃-dependent kinase PDK1 was shown to phosphorylate Thr308 of PKBα (15,16). Overexpression of PDK1 did not result in phosphorylation of Ser473, illustrating that the phosphorylation status of Ser473 is independent of Thr308. The kinase that controls Ser473 is still obscure but has been tentatively defined as PDK2. A first candidate for PDK2 is MAPKAP 2, which phosphorylates PKB in vitro (13). A second is integrin-linked kinase-1, which phosphorylates PKB at Ser473 in a PI-3K dependent manner (17).

Earlier studies favored the model in which PKB activation exclusively involves heterologous phosphorylation at both activatory sites via ‘third party’ kinases located upstream of PKBα. This was concluded from mutational studies in which an exchange of Thr308 to Asp resulted in an increased PKB activity level without inducing phosphorylation of Ser473 and vice versa (8, 18). Furthermore a kinase-inactive, membrane-targeted PKBα mutant was phosphorylated in L6 cells stimulated with insulin (18). A recent publication however, suggests that PKB is activated by autophosphorylation at the Ser473 site (19). This model describes a two step activation process of PKB. The initial phosphorylation occurs in the activation loop on Thr308 by PDK1 and consequently triggers phosphorylation on the Ser473 site by autophosphorylation; the postulated ‘third party’ PDK2 kinase is thus elusive.

Stimulation of blood platelets with α-thrombin triggers formation of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ (20,21), reflecting activation of PI-3K. The formation of these products is biphasic, showing a rapid accumulation of PtdIns(3,4,5)P₃ and a delayed rise in PtdIns(3,4)P₂. Also the activation of PKB is biphasic, suggesting that the first wave is triggered by PtdIns(3,4,5)P₃ and the second by PtdIns(3,4)P₂ (22). So far, there is little insight in the mechanisms that couple these changes to PKB.

In the present study we set to explore the activation of PKB in platelets in more detail, giving special emphasis to the dual phosphorylation of the enzyme by the upstream kinases. The RT-PCR shows that platelets contain mainly PKBα. Using site-specific antibodies against phosphorylated Ser473 (P-Ser473) and phosphorylated Thr308 (P-Thr308) on PKBα, we demonstrate that among the many platelet activating agents tested, only α-thrombin and thrombopoietin (TPO) induce phosphorylation and activation of PKBα. The activation by α-thrombin involves a PI-3K independent pathway mediated via Ca²⁺-ions and PKC, indicating that a Ca²⁺-dependent PKC-subtype regulates the PDK2-dependent PKB activation.

**Experimental procedure**
Materials—Indomethacin, phorbol 12-myristate 13 acetate (PMA), \(\alpha\)-thrombin, thrombopoietin (TPO), and Ponceau S were obtained from Sigma (St Louis, Mo, USA). The PAR1- (SFLLRN) and PAR4-specific (GYPGKF) activation peptides were from Biosynthesis (Lewisville, USA). Protifar was supplied by Nutricia, (Zoetermeer, The Netherlands). 1-(6-((17\beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl (U73122), 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (Ly294002), Gö6976 and 1,2-bis-(0-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Bisindolylmaleimide I (GF 109203x) and okadaic acid were provided by Boehringer (Mannheim, Germany). (5Z,13E,15S)-6, 9\(\alpha\)-epoxy-11\(\alpha\), 15-dihydroxy-prosta-5, 13-dien-1-oic acid, sodium salt (PGI2) was obtained from Cayman Chemicals (Boston, USA). New England Biolabs, Inc. (Beverly, MA, USA) provided the PKB\(\alpha\)-specific antibodies Phospho-Akt-Ser473 and Phospho-Akt-Thr308 and the horseraddish-peroxidase (HRP)-conjugated goat-anti-rabbit IgG. The antibodies recognizing phosphorylated as well as non-phosphorylated PKB\(\alpha\), -\(\beta\) and -\(\gamma\) were from From Santa Cruz (CA, U.S.A.). An antibody generated against the phosphorylated Thr641 in the turn motif of PKC\(\beta\)III, which cross reacts with the phosphorylated Thr450 of PKB, enhanced chemiluminescence reagent (ECL) and the Kodak X-OMAT blue X-ray films were obtained from NEN Life Science Products (Boston, USA). Nitrocellulose (Optitran BA-S 85) was obtained from Schleicher & Schuell (Dassel, Germany). Protein A sepharose beads were purchased from Pharmacia Biotech (Uppsala, Sweden). \([^{32}P]-\gamma\text{ATP} (3000 \text{ Ci/mmole})\) was obtained from New England Nuclear (Boston, USA). The PKB\(\alpha\) Immunoprecipitation Kinase Assay Kit was supplied by Upstate Biotechnology (Lake Placid, NY, USA). Dynabeads-M450 CD50 were obtained from Dynal (Oslo, Norway). All chemicals had a purity of more than 98%.

Platelet isolation—Venous blood was collected from medication-free volunteers (with informed consent) in 13 mM trisodium citrate (final concentration). The blood was centrifuged (200xg, 10 min at 20\(^\circ\) C) to yield platelet-rich plasma (PRP). Washed platelets were obtained by centrifugation of the PRP (200 x g, 10 min, at 20\(^\circ\) C), supplied with ACD and PGI2 (10 ng/ml) in order to prevent preactivation during the washing procedure. Platelets were resuspended in HEPES/Tyrode buffer (145 mM NaCl, 5 mM KCl, 1.0 mM MgSO\(_4\), 10 mM HEPES, pH 7.2) containing 5mM glucose and adjusted to 2\times10^{11} platelets/l. Prior to the experiments, platelets were left to rest at room temperature for 30 min. Gel-filtered platelets were prepared as described earlier (23).
All experiments were performed on indomethacin-treated platelets to exclude involvement of thromboxane A₂ (TxA₂). Therefore, platelets were preincubated with 30nM indomethacin (15 min, room temperature) prior to stimulation. Dose-response studies for PKB phosphorylation in platelets stimulated with α-thrombin, TPO and PMA were performed with 160 µl platelet suspension incubated for 10 min at room temperature without stirring. The reaction was stopped by adding 40 µl of a 5-fold concentrated sample buffer (625 mM Tris/HCl, pH 6.8, 50% glycerol, 5% SDS, 7.5 mM dithiothreitol, 0.05% bromphenol blue). The samples were mixed and stored on ice. For the determination of time-courses, 2 ml of platelet suspension was stimulated as described and samples of 160 µl were drawn. Experiments in the presence of bisindolylmaleimide I (5µM), Ly294002 (10µM), Gö6976 (10µM) and BAPTA-AM (10 µM/30 µM), reflect data obtained after 5 min preincubation with inhibitors at room temperature. Both agonists and enzyme modulators were added from 100-fold concentrated stock solutions; incubation conditions reflect final concentrations.

**RT-PCR from platelet cDNA** In order to identify the distribution of the PKB isoenzymes (PKBα, -β, -γ) in platelets, the following degenerated oligonucleotide primers from conserved regions of human PKB (α position 262-282, 1043-1063; β position 121-141, 935-975; γ position 63-83, 835-975) were used:

5’CTGG(C/A)GGCCA(C/A)G(G/A)TACTTCC3’ (sense) and 5’ CC(G/A)TC(T/C) TTGTCCAGCAT(G/T)AG3’ (antisense). Resulting PCR product: 665 bp. The common 5’ primer for all PKB subtypes was 5’CT(C/A)ATGCTGGACAA(G/A)GA(T/C)GG3’ and depending on the isoenzyme 5’CCACGTCTGCCAGTGATG3’ (PKBα), 5’GTGCTGCCACACAGATACCAG3’ (PKBβ) and 5’CCATGCAGTCCATACCCCATC3’ (PKBγ). Resulting PCR products: 354 bp (PKBα), 384 bp (PKBβ), 500 bp (PKBγ). mRNA was obtained from gel-filtered human platelets using the MACS mRNA isolation kit (Miltenyi Biotec, Germany) and reverse transcribed using the first-strand cDNA synthesis kit according to the manufacturer’s instructions (Pharmacia, Freiburg, Germany). Platelets were depleted from leukocytes using Dynabeads-M450 CD50 according to the manufacturer’s instruction. Before RT-PCR was performed, platelet cDNA was screened for contaminating cDNA derived from leucocytes. This control was performed by RT-PCR using primers against a leucocyte specific surface marker (24): HLA-DQb5 5’GTCTCAATTATGTCTTGGA3’ (sense) and HLA-DQb3 5’GCCACTCAGCATCTTGCT3’ (antisense). The resulting PCR product was 702 bp. Platelet cDNA was considered leucocyte-free when no PCR-product was amplified from platelet cDNA with HLA-DQb3/b5 primers after 35 PCR cycles. For amplification of genes encoding
PKB α, -β and -γ, 50 ng of platelet cDNA was used as a template. As positive control for the PKBγ primers cDNA from human thymus (Clontech, CA, U.S.A.) was used as a template. Amplification was carried out as described earlier (25). Briefly, the reaction was performed in 50 µl of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl2, with 200 µM of each dNTP, 100 pmol of each primer and 2U of Taq DNA polymerase (Gibco BRL, Gaithersburg, MA, USA). The following PCR protocol was performed with all primers: 94°C for 2 min, 55°C for 2 min, and 72°C for 5 min (1 cycle); 94°C for 30 s, 55°C for 1.5 min and 72°C for 40 s (34 cycles); 72°C for 7 min. After the RT-PCR, 10 µl of the products was separated on an 1 % agarose gel. PCR products were subcloned into the pGEM-5Zf(+) vector using the pGEMT-T vector system I (Promega, Mannheim, FRG). Recombinant plasmids were subjected to DNA sequencing using the RRDye Deoxy Terminator cycle sequencing kit (Perkin Elmer, Weiterstadt, Germany) with the sequencing primers Sp6 and T7.

Measurement of PKB phosphorylation-Platelet samples were boiled for 5 min at 96°C and proteins were separated on 12 % SDS-PAGE in 25mM Tris-base, 192.5 mM glycine, 0.1% SDS. Proteins were blotted onto nitrocellulose in a buffer containing 25 mM Tris, 192 mM glycine (pH 8.3) and 20 % methanol (v/v) using the BioRad2 wet blotting system. Protein-transfer was controlled by Ponceau S staining. Thereafter, the blots were destained in TBST (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 15 min. Nonspecific binding sites were blocked with 5 % Protifar in TBST for 30 min at room temperature and the blots were incubated overnight at 4°C with a polyclonal antibody raised against PKBα (1:4000), −β (1:4000), and -γ(1:4000) or against phosphorylated PKBα using Phospho-Akt Ser473 (1:1500, in TBST/5%Protifar) under continuous shaking. After three washes with TBST, the blots were probed with the HRP-conjugated goat anti rabbit IgG (1:2000 in TBST/5% Protifar) for 45 min at 4°C. After additional washing steps, the ECL-system was used to detect immunoreactivity on X-ray films. For analysis of Thr308 phosphorylation, the blots were stripped by a 30 min incubation in TBST supplied with 2 % SDS at 80°C. After extensive washing with aqua dest, the blots were subjected to the same procedure as described above, using polyclonal Phospho-Akt Thr308 antibody (1:1500) as first antibody. Since this antibody recognizes an aspecific protein band of 70 kDa, this band was taken as a control for lane loading. Quantification of the immuno positive bands was performed after scanning of the X-ray film using Image Quant software (Molecular Dynamics, Sunnyvale, USA).
In vitro kinase assay for PKB-The agonist-dependent activity of PKB was determined using a modification of the PKBα Immunoprecipitation Kinase Assay Kit from Upstate Biotechnology. Briefly, 250 µl washed platelets were stimulated with α-thrombin (0.5U/ml), TPO (50ng/ml) or PMA (0.5µM) for 10 min at room temperature. The reaction was stopped by addition of 2x concentrated lysis buffer (100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM Na3VO4, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 20 mM sodium β-glycerophosphate, 0.1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µM microcystin). The samples were snap-frozen in liquid nitrogen and stored at -70°C. Total PKBα was immunoprecipitated from the lysate with 4 µg of the antibody, recognizing non-phosphorylated as well as phosphorylated PKBα, coupled to protein-A sepharose beads in an end-over-end shaker overnight at 4°C. The enzyme/antibody/protein A-sepharose pellet was washed three times with 500 µl lysis buffer containing 0.5 M NaCl, twice with 500 µl washing buffer (50 mM TrisHCl, pH 7.5, 0.03% (w/v) Brij-35, 0.1mM EGTA and 0.1% (v/v) 2-mercaptoethanol) and twice with 100 µl assay dilution buffer (ADB) (10 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM sodium orthovanadate, 1 mM dithiothreitol). The pellet was resuspended in 10 µl of ice-cold ADB, 10 µl of 40 µM PKA inhibitor peptide (TYADFIASGRGTGRNNAI-NH3) and 100 µM PKB substrate peptide (RPRAATF), included in the kit. The reaction was started by adding 1 µCi/ml [32P]-γATP per sample. The mixture was incubated for 10 min at 30°C under continuous shaking. The proteins were precipitated with 40% trichloric acid and 40 µl aliquots were spotted onto phosphocellulose paper. The paper was washed three times in 0.75% phosphoric acid and once in acetone and the radioactivity was determined in a scintillation cocktail.

For in vitro phosphorylation experiments of PKBα by catalytic subunits of PKCα/β, two different experimental approaches were performed. First, PKBα was immunoprecipitated from resting platelets as described above and was incubated with 0.02 µg of catalytical PKC fragment in 40 µl of a buffer containing Tris/HCl (50 mM, pH 7.4) and 10 mM MgCl2. The reaction was started by addition of 4 µl [32P]-γATP (3000 mCi/mol). After 30 min at 30°C the reaction was stopped by addition of SDS sample buffer. Samples were subjected to 12 % SDS-PAGE and [32P] incorporation was determined by autoradiography. In the second approach 400 µl total lysate from resting platelets (2·10^7 platelets/ µl) was prepared as described above and incubated with purified PKC catalytical subunit (0.02 µg), PMA (0.5 µM), α-thrombin (0.5U/ml) or TPO (50 ng/ml). The phosphorylation reaction was started by addition of ATP (100 µM) at 30°C. At indicated time points samples of 55 µl were removed and added to new tubes containing
concentrated sample buffer. Phosphorylation of PKB on Ser473, Thr450 and Thr308 was determined in Western blot analysis as described above.

**Sequence analysis**

Sequences were analyzed using HUSAR 3.0 software package based on the sequence analysis software package 7.2 from the Genetic Group (Madison, WI).
Results

PKB isoenzymes in human blood platelets—We first identified the PKB isoenzyme distribution by RT-PCR and compared the results with the expression levels in Western blot analysis. Degenerated oligonucleotide primers against conserved sequence regions in the PH- and catalytic domain of rat and human PKB (α, β, γ) and PKCα−, -β and -γ-specific primers were employed using cDNA from human blood platelets as template. The primer combinations resulted in amplification of products with the expected sizes for the PKBα and PKBβ genes; no product for PKBγ was detected (Fig. 1A).

The PCR products amplified with degenerated and subtype-specific primers were cloned into a pGEM-T vector and subjected to DNA sequencing. Eighty percent of the analyzed clones obtained with the degenerated oligonucleotides showed 100% amino acid sequence homology with human PKBα and 20% of the clones showed 98% homology with human PKBβ. The PCR bands amplified with the PKBα− and PKBβ−specific primers were subjected to the same procedure and showed 100 % sequence homology with PKBα and PKBβ. No PCR product was obtained with the PKBγ−specific primers, indicating that PKCγ mRNA is not detectable in platelets. Interestingly, the PCR band amplified with PKBα primers was much stronger than the band of PKBβ. Since both primers have identical potencies, as confirmed by RT-PCR of the PCR products subcloned into the pGEM-T (data not shown), these results indicate that PKBα is the predominant PKB subtype in human platelets. This conclusion was supported by Western blot analysis showing a higher expression PKBα than PKBβ, whereas PKBγ was not detected (Fig. 1B).

α-Thrombin and TPO induce PKB-Ser473 phosphorylation in a dose- and time-dependent manner— In order to identify the mechanisms that initiate PKB phosphorylation, we used antibodies specific for P-Ser473, P-Thr450 and Thr308. From all agonists tested, including ADP (10 µM) and platelet activating factor (PAF, 5nM), only α-thrombin (0.1 U/ml) and TPO (20 ng/ml) triggered a rapid and sustained PKBα-Ser473 phosphorylation (P-Ser473).

Fig. 2A shows that increasing concentrations up to 0.3 U/ml α-thrombin were accompanied by a parallel rise in P-Ser473, resulting in a 4 fold increase at 0.1 – 0.2 U/ml α-thrombin. Higher concentrations revealed further phosphorylation, which did not reach saturation at 1.2 U/ml α-thrombin. Since in human platelets α-thrombin signals via PAR1 and PAR4 receptors, experiments were repeated with the PAR1 activating peptide SFLLRN and the PAR4-activating peptide GYPGKF. The PAR1-peptide (15µM) induced about 50% of the Ser473 phosphorylation observed with α-thrombin whereas the PAR4-peptide (500µM) failed to activate PKBα completely.
Simultaneous addition of both peptides induced the same PKBα activation as α-thrombin (data not shown). A much weaker Ser473 phosphorylation was induced by TPO with a 2.5-fold increase at 50 ng/ml (Fig. 2B). In contrast to α-thrombin, the effect by TPO was saturable and TPO doses up to 75 ng/ml did not further increase P-Ser473. Reprobing of the blots with anti P-Thr308 antibody showed that α-thrombin and TPO also raised the level of P-Thr308 and that the dose-response curves for both agonists were similar to those of P-Ser473 (data not shown).

To determine the kinetics of PKB phosphorylation, P-Ser473 and P-Thr308 were monitored over a time-range of 1-20 min. As depicted in Fig. 3A, α-thrombin caused a rapid increase in P-Ser473, showing a 3- (after 1 min) to 5-fold (after 10 min) increase, which remained stable for another 10 min. TPO initiated a slower and about 1-fold weaker phosphorylation compared with α-thrombin. Again, the changes in P-Thr308 were similar to those of P-Ser473 (Fig. 3B). In order to examine whether the third phosphorylation site, Thr450, was affected by the agonists, the blots were reprobed with a polyclonal antibody recognizing phosphorylated Thr450. As depicted in Fig. 3C, no changes in the basal phosphorylation of Thr450 were detected. These results illustrate that α-thrombin is a more potent activator of PKB-phosphorylation than TPO and suggest that both agonists start different signalling pathways that lead to concurrent phosphorylation of Ser473 and Thr308.

**α-Thrombin and TPO initiate PKB phosphorylation via different signalling pathways** - Earlier studies have shown that PI-3K is the main upstream regulator of PKB (5,6,7). Fig. 4A illustrates that LY294002, an inhibitor of PI-3K, inhibited the rise in P-Ser473 induced by both agonists, but there was a notable difference between α-thrombin and TPO. LY294002 reduced the α-thrombin-induced Ser473 phosphorylation by about 60% whereas the inhibition of TPO-induced Ser473 phosphorylation was almost complete. Hence, Ser473 phosphorylation induced by α–thrombin was partly independent of PI-3K, a property not seen with TPO. Interestingly, this difference was not seen at the level of Thr308, where LY294002 inhibited both the α-thrombin- and TPO-induced phosphorylation almost completely (Fig. 4B). Thus, both α-thrombin and TPO initiate the phosphorylation of Thr308 via PI-3K. In addition, there is a second, PI-3K-independent pathway that is initiated exclusively by α-thrombin and is responsible for about 60% of the phosphorylation of Ser473. To investigate whether differences in Thr308 and Ser473 phosphorylation were caused by selective dephosphorylation by phosphatases, studies were repeated in the presence of okadaic acid (1 µM), an inhibitor of the phosphatases 1 (PP1) and 2A (PP2A), since PP2A can dephosphorylate Ser473 (26). Okadaic acid did not change the low phosphorylation levels of PKBα in resting platelets or the increase in P-Thr308.
and P-Ser473 induced by TPO and α-thrombin. Also, the different phosphorylation seen in the presence of the PI-3K inhibitor remained unchanged. The difference between Thr308 and Ser473 phosphorylation is therefore the result of different regulation by PDK1/2 (data not shown).

A major difference between the signalling pathways initiated by the TPO (cMpl)-receptor and the thrombin (PAR1,4)-receptor is the involvement of phospholipase Cβ. This enzyme is activated by seven transmembrane receptors coupled to trimeric G-proteins following release of Gα and βγ subunits. Hence, α-thrombin but not TPO activates PLCβ (1/2) and this activation is independent of PI-3K (27,28). Fig. 5A depicts the effect of the PLCβ2 inhibitor U73122 (29). Whereas the TPO-induced Ser473 phosphorylation was not disturbed, the inhibitor sharply reduced P-Ser373 levels raised by α-thrombin, resulting in an 80% inhibition after 10 min.

PKCs activate PKB by phosphorylation on Ser473-To clarify which elements downstream of PLCβ2 contributed to Ser473 phosphorylation, we evaluated the role of PKC (30, 31). Typical PKC isoforms (PKCα, -β, -γ) are regulated by Ca²⁺ and phospholipids. Their activation can be mimicked by the phorbol ester PMA and inhibited by the phorbol ester analogue bisindolylmaleimide I (31). As illustrated in Fig. 5B, bisindolylmaleimide I inhibited the α-thrombin-induced phosphorylation of Ser473 by 70%. In contrast, TPO-dependent Ser473 phosphorylation was not changed by the inhibitor. These results suggest that the PI-3K-independent rise in P-Ser473 induced by α-thrombin results from activation of PKC.

This conclusion was substantiated in experiments with PMA. Already a low concentration of PMA (0.05µM) increased the level of P-Ser473 and a maximum was reached at 1.0 - 1.5 µM PMA (Fig. 6A, upper panel). These high concentrations were used to provoke the phosphorylation of Thr308, but no increase by PMA was observed (Fig. 6A, lower panel). The specificity of PMA was confirmed by inclusion of the PKC inhibitor bisindolylmaleimide I, which completely blunted the effect by PMA (Fig. 6B). An earlier report described that PMA increases the level of PtdIns(3,4,5)P3 via activation of p85/110 PI-3K (32). As this might interfere with the present experiments, studies were repeated in the presence of the PI-3K inhibitor LY294002. PMA induced the same phosphorylation of Ser473 and not of Thr308, indicating that PI-3K was not involved (data not shown).

To identify which PKC subtype increased P-Ser473, we investigated the effect of Gö6976, an inhibitor of the classical (33), Ca²⁺-dependent PKCs (PKCα, PKCβ1/2) and BAPTA-AM, a Ca²⁺ chelator. As shown in Fig. 7A, Gö6976 abolished 60% of the PMA response. Approximately the same inhibition was observed when calcium ions
were scavenged by BAPTA-AM. As expected, these treatments had no effect on the level of P-Thr308, confirming that PKC subtypes did not take part in the regulation of Thr308 phosphorylation. Similar results were obtained in platelets stimulated with α-thrombin, with the exception that the complete inhibition by bisindolylmaleimide I observed in PMA-stimulated platelets was incomplete following stimulation with α-thrombin (Fig. 7B). In TPO-stimulated cells, the PKC modulators neither changed the phosphorylation of Ser473 or that of Thr308 (Fig. 7C).

Relation between PKB phosphorylation and activation.- In order to investigate whether the PMA-induced phosphorylation of Ser473 affected the enzyme activity, PKBα was immunoprecipitated from stimulated platelets and the kinase activity was measured with an artificial substrate. Fig. 8 shows that PMA-treatment increased PKBα activity with 50 % above basal levels. This was a rather moderate increase compared with α-thrombin and TPO, which induced increases to 500 and 250 % respectively. These results raise the question whether Ser473 is directly or indirectly phosphorylated by PKCs. To investigate this, PKBα was immunoprecipitated from unstimulated platelets and [32P] incorporation was measured after addition of catalytically active PKCα/β subunit in the presence of MgCl2 and [32P]-γATP. Although incorporation of [32P] into the PKC substrate histone 1 was observed, no phosphorylation of PKB was measured in either radiography or Western blot analysis using Ser473 antibodies (data not shown). To evaluate whether this negative result was caused by the absence of one or more cofactors, studies were repeated in platelet lysate. Active PKC fragments induced an increase in phosphorylation in Ser473 with a velocity and extend that was in the range of PMA induced Ser473 (Fig. 9A). The potency of the PKC fragment to phosphorylate Ser473, Thr308 and Thr450 was compared with PMA, α-thrombin and TPO. The active PKC fragment exclusively induced Ser473 phosphorylation as did PMA without concurrent Thr308 and Thr450 phosphorylation (Fig. 9B). Again, α-thrombin and TPO increased both Ser473 and Thr308 phosphorylation levels. These findings suggest that phosphorylation and activation of PKB by PKC require additional factors present in platelet lysate.

Taken together, our results illustrate, that selective phosphorylation of Ser473 is insufficient for full PKB activation. They also indicate that α-thrombin, TPO and active PKC fragments or PMA induce distinct phosphorylation patterns that enable fine-regulation of PKB activity.
Discussion

The main conclusions from this study are: (i) α-Thrombin and TPO activate PKBα via simultaneous phosphorylation of Ser473 and Thr308; (ii) α-Thrombin signals to PKBα via two pathways, one involving PI-3K and one involving PLCβ2. In contrast, TPO signals only via a PI-3K-dependent pathway; (iii) The PLCβ2-dependent pathway to PKBα is mediated via a calcium-dependent PKC subtype (PKCα / PKCβ). (iv) Direct PKC-activation by PMA increases PKBα activity by selective phosphorylation of Ser473, (v) this activity of PKC depends on an unknown cofactor in platelet lysate. Together these findings suggest at least four possible conformations of PKB each differing in number and extent of phosphorylated Ser473 and Thr308 and concomitant enzyme activity.

α-Thrombin is a serine protease and the most potent activator of human platelets. It induces shape change, secretion and aggregation via activation of protease-activated receptors or PAR-receptors by proteolytic cleavage of the NH2-terminal receptor exodomain. The new NH2-terminus serves as a tethered peptide ligand, which intramolecularly binds to the body of the receptor, thereby initiating signal processing (34, 35). Human platelets use a dual PAR-receptor system, consisting of a high affinity PAR1-receptor which is activated at low thrombin concentrations (1 nM, or 0.09 U/ml) and a low affinity thrombin receptor (PAR4), which is activated at 30 nM (2.7 U/ml). Both receptors are required for optimal thrombin-induced aggregation and secretion. PAR(1,4) couples to the trimeric G-proteins Gq and Gi, enabling Gq and βγ subunits to activate phospholipase C-β1/2, which in turn generates phosphatidylinositol(1,4,5)P3 (IP3) and diacylglycerol. IP3 increases cytosolic Ca2+ via mobilization of intracellular storage sites and store-operated Ca2+ influx and together with diacylglycerol activates the classical PKC-subtypes. α-Thrombin also activates PI-3K. Although this pathway is still elusive, there is evidence for a role of non-receptor tyrosine kinases, such as p72syk and p60src resulting in activation of the p85/110 form of PI-3K. Besides p85/110, a second type of PI-3K, called PI-3Kγ contributes to the α-thrombin-induced PtdIns(3,4,5)P3 formation in human platelets. In contrast to p85/110, this latter kinase subtype is activated by βγ subunits released from trimeric G-proteins rather than tyrosine phosphorylated proteins (32).

The present data show that α-thrombin activates PKBα via simultaneous phosphorylation of Ser473 and Thr308 and that it does not affect the basal phosphorylation of Thr450. A similar pattern is induced by a combination of PAR1- and PAR4-activating peptides. Interestingly, the PAR1-peptide alone increased Ser473 and Thr308 phosphorylation to only 50% of the response induced by α-thrombin, whereas the PAR4-activating peptide failed to activate PKB
completely. These findings support the concept that both receptor subtypes must be activated for full induction of PKB activity (13). The fact that the dose-response curve for α-thrombin-induced PKBα phosphorylation did not reach saturation at 1 U/ml α-thrombin, suggests that higher agonist concentrations are required to saturate the PAR4-receptor. This dual mechanism of PKB regulation might reflect a sensitive means for platelets to adjust the PKB activity to small changes in the α-thrombin concentration.

Recently, it was reported (22) that α-thrombin induced a biphasic activation of PKB in stirred platelet suspensions and that this activation completely depended on PI-3K. The two phases were thought to result from an initial formation of PtdIns(3,4,5)P3 during the onset of aggregation and a later formation of PtdIns(4,5)P2 during the postaggregational phase. The present data do not show such a biphasic PKB activation pattern, possibly because our incubation conditions (unstirred, room temperature) were chosen to prevent aggregation. In line with the previous study is the predominant role of PI-3K in the regulation of PKB. Both in α-thrombin- and in TPO-stimulated platelets the phosphorylation of Thr308 on PKB was completely abolished by the PI-3K inhibitor LY294002. Also phosphorylation of Ser473 depended completely on PI-3K in TPO-stimulated platelets. In α-thrombin-stimulated cells, the inhibition by LY294002 was incomplete, revealing a second, PI-3K-independent activation route, which specifically contributed to the phosphorylation of Ser473.

TPO is a cytokine with a NH2-terminus with high homology to erythropoietin and a unique glycosylated C-terminal domain. It is the ligand for the cMpl-receptor, a transmembrane, non-receptor kinase and a member of the superfamily of cytokine receptors (35). Expression of this receptor is restricted to haematopoietic progenitor cells, megakaryocytes and platelets (36, 37). c-Mpl-deficient mice have a more than 85% reduced platelet count and a decreased number of bone marrow megakaryocytes compared to controls, indicating that TPO is the major regulator for proliferation and maturation of megakaryocytes as well as platelet formation. In physiological concentrations (<100 ng/ml) TPO fails to induce aggregation or secretion but a short TPO-platelet contact suffices to increase the sensitivity of platelets to α-thrombin or ADP (38). TPO induces rapid tyrosine phosphorylation of the cMpl-receptor and of Shc, JAKs and the latent transcription factor STAT1 (39). Phosphorylated c-Mpl and Shc function as potential binding sites for proteins that contain Src homology 2 (SH2) domains or phosphotyrosine-binding domains. Shc takes part in activation of the Ras pathway by binding to Grb2 and SOS. It also activates p85/110 PI-3K by phosphorylating the regulatory p85 subunit (40).
The observation that TPO induces a parallel phosphorylation of Thr308 and Ser473 whereas in platelets treated with a PKC-inhibitor α-thrombin triggers more Thr308 phosphorylation than Ser473 phosphorylation, suggests the involvement of different PI-3K subtypes (Fig. 10). α-Thrombin is known to activate p85/110 PI-3K and PI-3Kγ (32). The p85/110 PI-3K is a heterodimer consisting of a regulatory non-catalytic p85 subunit and a catalytic p110 subunit. Activation occurs by the tyrosine kinases p72Syk and/or p62Src, which recruit the cytosolic p85 subunit to the plasmamembrane. Once tyrosine phosphorylated, it forms an active, PtdIns(3,4,5)P3 generating complex with the catalytic p110 subunit. In contrast, PI-3Kγ does not associate with a p85 adaptor subunit, but binds to βγ subunits derived from Gαi or Gαq-proteins in the plasmamembrane which trigger the activation. In neutrophils, these PI-3K subtypes differ also in sensitivity for the inhibitor wortmannin, with p85/110 PI-3K showing a higher sensitivity than PI-3Kγ (32). In the concentrations used in this study, TPO fails to raise Ca²⁺ or to activate PKC in platelets and liberation of βγ subunits is therefore unlikely. Hence, TPO triggers the parallel phosphorylation of Thr308 and Ser473 entirely through p85/p110 PI-3K. A similar activation might occur by α-thrombin, which would account for the remaining Ser473 phosphorylation in platelets treated with a PKC-inhibitor. Assuming a similar stoichiometry as in TPO-stimulated cells, p85/p110 PI-3K would account for the same extent of Thr308 phosphorylation in these cells. The finding that P-Thr308 is much higher than P-Ser473 in the presence of a PKC-inhibitor might be the result of a second route to P-Thr308, possible initiated by PI-3Kγ. An alternative explanation is that PI-3Kγ signals to Thr308 together with a second type of p85/p110 PI-3K that initiates selective phosphorylation of Ser473. A better insight in the coupling between PI-3K subtypes and PKB phosphorylation awaits the development of PI-3K subtype specific antibodies.

In literature there is support for both autonomous and heterologous phosphorylation of PKB. In a purified system, initial Thr308 phosphorylation by PDK1 triggers autophosphorylation of Ser473 and maximal PKB activation (19). Our data, showing a simultaneous phosphorylation of both activatory sites, rather suggest that activation of PKBα in human platelets mainly occurs via heterologous phosphorylation by different upstream kinases regulating phosphorylation of either Thr308 or Ser473. Moreover, both sites are phosphorylated via different signaling pathways, depending on the agonist. There is evidence for a role of at least three different PDKs, one Thr308-kinase and at least two Ser473-kinases. The PI-3K dependency of the Thr308 phosphorylation in both α-thrombin- as well as TPO-stimulated platelets is in line with other studies suggesting PDK1 as the upstream Thr308 kinase (13, 16).
contrast, Ser473 phosphorylation showed a partial sensitivity for LY294002. This might be explained either by a
dual phosphorylation by one or two independent Ser473 kinases instead of autophosphorylation as suggested in the
studies of Toker and Newton (19).

Platelets express the PKC subtypes α,β,δ,η,θ,ζ (29,30). These subtypes differ in their sensitivity to signalling
molecules of the phospholipase Cβ pathway, such as diacylglycerol, calcium ions and phosphatidylinserine which
activate PKCα and -β, diacylglycerol and phosphatidylinserine which activate PKCδ, −η and -θ and
phosphatidylinserine alone, an activator of PKC-ζ. In addition, PtdIns(3,4,5)P3 and PtdIns(3,4)P2 increase the activity
of the calcium-independent PKCδ and -ζ (41). Activation of PKC is a key step in the initiation of platelet functions,
e.g. exposure of ligand binding sites on integrin αIIbβ3, enabling fibrinogen binding and aggregation, extrusion
of secretory granules leading to surface exposure of activation markers like P-selectin, thrombospondin, and the internal
pool of αIIbβ3. The present results indicate that one of the calcium-dependent PKC subtypes, PKCα and/or PKCβ,
activates PKBα via phosphorylation of Ser473, independently of PI3-kinases. Evidence for this comes from the
observation that (i) activation of PKCs by PMA increases the level of P-Ser473 and PKBα activity; (ii)
bisindolylmaleimide I selectively inhibits α-thrombin- and PMA-induced Ser473 phosphorylation, (iii) Gö6976, the
inhibitor for the calcium-dependent PKCs and (iv) the chelator of calcium ions by BAPTA lowers P-Ser473 to the
same range as bisindolylmaleimide I. One might question if the effects of BAPTA on P-Ser473 can exclusively point
to the involvement of typical PKCs in this process, as other calcium-dependent enzymes might be affected as well.
Literature data show that calcium-dependent regulation of PKBα is performed by Ca2+/calmodulin-dependent kinase
kinase 2 and occurs on the level of Thr308 phosphorylation (42). This observation is in contrast with the present
data, which show no calcium-dependency of Thr308 phosphorylation. Furthermore, Ser473 phosphorylation induced
by PMA was inhibited by BAPTA, indicating that a role for calcium-dependent PKC is likely.

Earlier studies (43) reported that PKC may activate PI-3K, which could enhance the PKB phosphorylation observed
in the present studies. However, similar rises in P-Ser473 were seen in PMA-treated platelets in the absence and
presence of LY294002 and such a feedback activation appears unlikely. The question remains whether PKCs directly
or indirectly phosphorylate PKBα.

Analysis of the full-length PKBα amino acid sequence PROSITE for putative PKC phosphorylation sites indicate 6
putative phosphorylation sites for PKCs: Thr21, Thr65, Thr172, Ser266, Thr305 and Ser463. Thus, Thr308 and
Ser473 investigated in the present study are not typical PKC phosphorylation sites and also the adjacent amino acids differ from those normally seen in these domains. This suggests that Ser473 phosphorylation induced by PMA is not the result of direct phosphorylation by PKC.

However, we failed to monitor direct phosphorylation of PKB by a catalytically active PKC fragment in vitro. Instead, we observed Ser473 phosphorylation by the PKC fragment when experiment were performed in platelet lysate. We therefore conclude that PKCs need an additional, yet unidentified compound or cofactor, present in platelet total lysate, to complete the PKBα activation process by phosphorylation of Ser473.

Together, the present findings illustrate that PKB can undergo different degrees of phosphorylation, depending on the type of platelet agonist and that phosphorylation of Ser473 and Thr308 can occur independently. The constitutively phosphorylated Thr450 does not contribute to PKB activation. This enables the platelet to fine-regulate PKB activity. The contribution of the PKC-dependent Ser473 phosphorylation may be responsible for the faster PKB activation seen after stimulation with α-thrombin compared with TPO. In addition, PKB subtypes are known to form homo- and hetero-trimers, which may further expand the possibilities for fine-tuning PKB activity.

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Footnotes

The abbreviations used are:

GLUT, glucose transporter; GSK, glycogen synthase kinase; MAPKAP, mitogen-activating kinase protein kinase kinase; PCR, polymerase chain reaction; PDK, phosphoinositide-dependent kinase; PI-3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; PMA, β-phorbol myristate acetate; PtdIns, phosphatidylinositol; SH2, src-homology 2; TPO, thrombopoietin; PAR, protease activated receptor; PROSITE (www.cbs.dtu.dk/databases/PhosphoBase).
Figure legends

**Fig 1. RT-PCR with PKB primers**

Expression of PKB genes in platelets (A). mRNA isolated from leukocyte-depleted platelets was subjected to RT-PCR analysis by using degenerate primers corresponding to common sequence motifs of human PKBα, -β and -γ (PKBall) and primers specific for human PKBα, PKBβ and PKBγ. The PCR products were size fractionated on an agarose gel, resulting in bands of 354 bp (PKBα), 384 bp (PKBβ) and 665 bp (PKB all). No band for PKBγ (expected size 500 bp) was found. In parallel experiments, total platelet lysate (25 µg/lane) was subjected to SDS-PAGE and developed with PKBα, -β and -γ specific antibodies in Western Blot analysis (B). Bands were quantified and expressed as pixels/µg protein.

**Fig. 2. Concentration-dependent Ser473 phosphorylation on PKBα induced by α-thrombin and TPO.**

Washed, indomethacin-treated platelets, were incubated with different concentrations of α-thrombin (A) (closed circles) or TPO (B) (open circles) for 10 min at room temperature without stirring and P-Ser473 was determined by Western blot analysis. Data are expressed as percentage of the Ser473 phosphorylation in resting platelets and are means ± SD, n=4.

**Fig. 3. Kinetics of Ser473 and Thr308 phosphorylation.** Platelets were stimulated for 10 min with 0.5 U/ml α-thrombin (closed circles) or 50 ng/ml TPO (open circles). First, Ser473 phosphorylation was quantified (A), then the blots were stripped and reprobed with the P-Thr308 (B) and P-Thr450 (C) antibody. Besides the 60kDa protein band, which represents phosphorylated Thr308 on PKBα, the P-Thr308 antibody also recognized an unidentified 70 kDa protein band, which was taken as a control for lane loading. Data are means ± SD, n=5. Further details as in figure 2.

**Fig 4. Role of PI-3K in PKBα phosphorylation.** Platelets were stimulated with α-thrombin (0.5 U/ml) or TPO (50 ng/ml) for 10 min. Prior to stimulation, platelets were incubated without or with LY294002 (10 µM, 10 min). The phosphorylation of PKB was analyzed with Phospho-Akt Ser473 (A) and Phospho-Akt Thr308 (B). Data are
expressed as percentage of controls (in the absence of inhibitor) and are means ± SD, n=4. Further details as in figure 2.

**Fig 5. Role of PLCβ2 and PKC in PKBα phosphorylation.** Platelets preincubated with U73122 (10 µM, 10 min; panel A) or bisindolylmaleimide I (5 µM, 10 min; panel B) were stimulated with α-thrombin (0.5 U/ml, closed circles) or TPO (50ng/ml, open circles) for the indicated time periods and P-Ser473 was determined. The inhibitors did not affect agonist-induced Thr308 phosphorylation (data not shown). Data are means ± SD, n=5. Further details as in figure 2.

**Fig. 6. Concentration-dependent phosphorylation of PKBα by PMA.** PMA (0.5 nM - 15 µM) -stimulated platelets were analyzed for P-Ser473 and P-Thr308 (A). The specificity of the PMA effect was verified in parallel experiments with platelets preincubated with bisindolylmaleimide I (5 µM, 10 min) before stimulation with 0.5µM PMA (B). The upper panel depicts a Western Blot developed with the P-Ser473 antibody and is representative for 4 independent experiments. Data in lower panels are expressed as % of control (no agonist) and are means ± SD, n=4. Further details as in figure 2.

**Fig. 7. PKC isoenzymes involved in PKBα phosphorylation.** Platelets were stimulated with (A) PMA (0.5µM), (B) α-thrombin (0.5 U/ml) and (C) TPO (50 ng/ml) without or with bisindolylmaleimide I (bisindo I ; 5µM), Gö6976 (10 µM) or BAPTA-AM (10 µM) for 10 min. Thereafter, the levels of Ser473 and Thr308 phosphorylation were determined in Western Blot analysis. The phosphorylation in platelets treated with agonist in the absence of inhibitor was defined as 100 %. Data are expressed as percentage of control and are means ± SD, n=5. Further details as in figure 2.
**Fig. 8 PKBα activity induced by PMA, α-thrombin and TPO**

Platelets were stimulated with PMA (0.5 µM), α-thrombin (0.5 U/ml) and TPO (50 ng/ml) for 10 min. The reaction was stopped by adding icecold lysis buffer. PKBα was immunoprecipitated from the Triton-100 soluble platelet lysate and the PKB activity was measured as described in ‘Experimental Procedures’. Control data reflect PKB activity in resting platelets and were defined as 100%. Results are expressed as % of control and are means ± SD, n=3. Further details as in figure 2.

**Fig. 9 In vitro phosphorylation of PKBα by purified catalytic subunit of PKCα/β**

Phosphorylation of PKB was performed in lysate of non-stimulated platelets. The reaction was initiated by addition of purified catalytic PKC subunits of PKCα/β and ATP (100 µM). The reaction was stopped after incubation at 30°C at the indicated time-points by addition of SDS sample buffer. Phosphorylation of PKB was monitored in Western blot analysis. The upper panel (A) depicts a representative experiment of a time-dependent phosphorylation of PKBα at Ser473 induced by the PKC catalytic subunits. Similar results were obtained in 4 independent experiments. In the lower panel (B) PKB phosphorylation on Ser473, Thr450 and Thr308 induced by PKC subunit was compared with that induced by PMA (0.5 µM), α-thrombin (0.5 U/ml) and TPO (50 ng/ml). The depicted blots are representative for 3 independent experiments.

**Fig. 10 Schematic model for the regulation of PKBα in platelets**

Model depicting regulation of PKB in platelets. TPO binds to the cMpl receptor and activates p85/p110 PI-3K possibly via a pathway involving ras and Shc. This results in PKBα activation by dual phosphorylation on Thr308 and Ser473 by PDK1 and PDK2, respectively. α-Thrombin activates the PAR1 and PAR4 receptors and initiates two pathways, one leading to activation of p85/p110 PI-3K possibly via p72Syk and P60Src and further PKB activation as observed with TPO, and a second leading to activation of PI-3Kγ and PLCβ possibly via βγ subunits released from trimeric G-proteins. PI-3Kγ initiates Thr308 phosphorylation via PDK1 and PKCβ activates calcium-dependent PKC subtypes that phosphorylate Ser473. For more details, see text.
A

P-Ser473

PMA [μM]

0 0.005 0.05 0.5 1.0 1.5

P-Thr308

PMA [μM]

0 0.005 0.05 0.5 1.0 1.5

B

| time [min] | 10 | 1 | 3 | 5 | 10 | 20 | 10 | 1 | 3 | 5 | 10 | 20 |
|-----------|----|---|---|---|----|----|----|---|---|---|----|----|
| PMA       | -  | - | + | + | +  | +  | +  | - | - | - | -   | -   |
| bisindo I | -  | - | - | - | +  | +  | +  | - | - | + | +   | +   |

![Graph A](image1)

![Graph B](image2)
Dual regulation of platelet protein kinase B
Christine Kroner, Kurt Eybrechts and Jan-Willem N. Akkerman

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