Dephosphorylation of the focal adhesion protein VASP in vitro and in intact human platelets

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Abstract The focal adhesion protein VASP, a possible link between signal transduction pathways and the microfilament system, is phosphorylated by both cAMP- and cGMP-dependent protein kinases in vitro and in intact cells. Here, the analysis of VASP dephosphorylation by the serine/threonine protein phosphatases (PP) PP1, PP2A, PP2B and PP2C in vitro is reported. The phosphatases differed in their selectivity with respect to the dephosphorylation of individual VASP phosphorylation sites. Incubation of human platelets with okadaic acid, a potent inhibitor of PP1 and PP2A, caused the accumulation of phosphorylated VASP indicating that the phosphorylation status of VASP in intact cells is regulated to a major extent by serine/threonine protein phosphatases. Furthermore, the accumulation of phosphorylated cAMP-dependent protein kinase substrate(s) appears to account for inhibitory effects of okadaic acid on platelet function.

Key words: Protein serine/threonine phosphatase; Okadaic acid; cAMP-/cGMP-dependent protein kinase

1. Introduction

Platelet function is regulated by a number of vasoactive agents. Whereas agonists like thrombin, thromboxane A_2 and ADP activate platelets resulting in shape change, adhesion, aggregation and degranulation [1], platelets are inhibited by cAMP- and cGMP-elevating agents such as prostaglandins E_1 and I_2 or nitrovasodilators [2,3]. The 46/50 kDa focal adhesion and microfilament associated [4] vasodilator-stimulated phosphoprotein (VASP) is a common substrate of cAMP-dependent and cGMP-dependent protein kinases (cAK, cGK) not only in human platelets but also in a variety of other cell types [4–9]. VASP phosphorylation correlates very well with the inhibition of platelet aggregation [2,6,8] and in particular with the inhibition of fibrinogen binding to glycoprotein IIb–IIIa [10]. Recent data suggest that VASP is an important link between signal transduction pathways and the microfilament system [11,12].

Three cAK/cGK VASP phosphorylation sites have been identified (Ser^{157}, Ser^{239}, Thr^{278}) [13,14]. All three sites are used by both protein kinases, but with different priority. In vitro, Ser^{239} is the phosphorylation site preferred by cGK while cAK first phosphorylates Ser^{157}. Phosphorylation of Ser^{157} causes the phosphorylation-induced mobility shift of VASP in SDS-PAGE from 46 kDa to 50 kDa. Consequently, transient phosphorylation of the 46 kDa VASP species is observed only with cGK but not with cAK [8,13].

The phosphorylation state of proteins is not only regulated by protein kinases but also by protein phosphatases (PP). In platelets, several different protein phosphatases have been identified, namely protein serine/threonine phosphatases type 1, type 2A [15,16], type 2B [17,18] as well as protein tyrosine phosphatases [19].

Studies with the potent and specific serine/threonine protein phosphatase inhibitors okadaic acid, calyculin A and tautomycin [20,21] demonstrated that these phosphatase inhibitors affect platelet function. All three phosphatase inhibitors reduced the receptor-mediated Ca^{2+}-influx into human platelets [22]. Earlier, okadaic acid was shown to inhibit platelet aggregation and other events associated with thrombin-induced platelet activation [15,23,24]. Also, effects on protein phosphorylation after treatment of cells with okadaic acid or calyculin A have been observed [15,23,25,26]. However, the identities of most phosphoproteins, affected by individual protein phosphatases and/or phosphatase inhibitors, are still unclear. Therefore, the identification of endogenous serine/threonine phosphatase substrates is mandatory in order to dissect the physiological functions of the different serine/threonine protein phosphatases.

In this study, we investigated the dephosphorylation of the well characterized cyclic nucleotide-dependent protein kinase substrate VASP by serine/threonine protein phosphatases in vitro and in intact human platelets. A preliminary account of this work has been presented in abstract form [27].

2. Materials and methods

2.1. Protein purification

The catalytic subunits of PP1 and PP2A from rabbit skeletal muscle, PP2B from bovine brain and PP2C from rat liver were purified as described [28]. Catalytic subunit of cAK, cGK and VASP were purified essentially as described [7], with some modifications as indicated [4].

2.2. Phosphorylation of purified VASP

VASP (2.9 μM) was incubated for 30 min at 30°C in buffer A (50 mM Tris-HCl pH 7.2, 1 mM dithioerythritol, 5 mM $MgCl_2$, 0.01% bovine serum albumin) containing 100 μM [γ-^{32}P]ATP (44,4 GBq/mmol; Amersham Buchler, Braunschweig, Germany) and 150 nM of cAK catalytic subunit. Under these conditions about 2–3 mole of phosphate per mole of VASP are incorporated [7,29]. ^{32}P-phosphorylated VASP was separated by one- or two-dimensional gel electrophoresis and detected by autoradiography. Autophosphorylation of cAK or cGK does not interfere with the analysis of VASP phosphorylation [7].

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Abbreviations: cAK, cAMP-dependent protein kinase; cGK, cGMP-dependent protein kinase; OA, okadaic acid; PP1, protein phosphatase type 1; PP2A, PP2B, PP2C, protein phosphatase type 2A, 2B and 2C; Sp-5,6-DCl-cBiMPS, Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-monophosphoro-thioate; w/o, without.

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2.3. Dephosphorylation of purified VASP

Following the phosphorylation of VASP, the catalytic subunit of cAK was inhibited by 10 μM PKI-peptide (TTYADFIASGRTGRRNAIHD; [30]). Traces of PBS (from purified VASP) which inhibit the phosphatases were removed by centrifugation through a MicroSpin S-200 HR column (Pharmacia, Freiburg, Germany), pre-equilibrated with buffer B (buffer A containing 0.03% (w/v) Nonidet P40).

Phosphorylated VASP (2.3 μM) was incubated at 30°C in buffer B (which also contained 0.05 mg/ml calmodulin/0.5 mM $CaCl_2$ for PP2B or 20 mM $MgCl_2$ for PP2C) with 0.3–0.5 μM of the respective phosphatase. At the time points indicated, aliquots were removed and the reaction was terminated by the addition of an SDS stop solution (for SDS-PAGE analysis) or by the addition of lysis buffer (9 M urea, 2% (w/v) Nonidet P40, 2% (v/v) 2-mercaptoethanol, 0.8% (w/v) ampholines pH 3–10) (for two-dimensional gel electrophoresis).

2.4. Incubation of washed platelets with okadaic acid, Sp-5,6-DCl-cBiMPS and Iloprost

For the preparation of washed human platelets, venous blood from healthy volunteers was collected into citrate buffer (20 mM sodium citrate, 3 mM EDTA pH 5.5) and centrifuged (300 × *g* for 10 min). Platelets of the resulting supernatant (platelet rich plasma) were pelleted at 420 × *g* for 20 min and resuspended in modified Tyrode's buffer (10 mM HEPES pH 7.4, 2.7 mM KCl, 137 mM NaCl, 5 mM glucose, 1 mM EDTA). This step was repeated once, and platelets were resuspended at a concentration of 1×10^9 cells/ml. After a 30 min preincubation at 20°C, platelet aliquots were incubated with different concentrations of okadaic acid (ammonium salt; LC Laboratories) for 40 min at 37°C. After this incubation, platelets were rapidly pelleted by centrifugation at 8,200 × *g* for 10 s, and the pellet was resuspended in lysis buffer for two-dimensional gel electrophoresis. In other experiments, washed platelets were similarly incubated with 5 nM Iloprost (a stable analogue of prostaglandin I_2; kind gift of Schering, Berlin, Germany) or 0.2 mM Sp-5,6-DCl-cBiMPS (Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-monophosphorothioate; a membrane permeable cAMP-analogue; Biolog, Bremen, Germany).

2.5. Immunoblotting

Immunoblotting was done as described [4], except that antibodies bound to immobilized ^{32}P-labeled VASP were detected by the enhanced chemiluminescence (ECL) detection method (Amersham Buchler, Braunschweig, Germany).

2.6. Two-dimensional gel electrophoresis

For isoelectric focusing, Immobiline dry strips pH 3–10.5 (Pharmacia, Freiburg, Germany) were pre-swollen overnight in rehydratization-solution (8 M urea, 0.5% (w/v) Nonidet P40, 10 mM dithiothreitol). Isoelectric focusing was carried out at 15°C for 24,700 Vh, with 10 mM glutamic acid as anode buffer and 10 mM lysine as cathode buffer. After focusing, gel strips were equilibrated twice for 15 min in a buffer containing 50 mM Tris-HCl pH 6.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 1% (w/v) dithiothreitol, 0.3% (w/v) Bromophenol blue and then applied to an SDS slab gel. As internal pI standards, carbonic anhydrase II from bovine erythrocytes (pI 5.4; Sigma) and myoglobin from horse heart (pI 6.8 and 7.2; Sigma) were used.

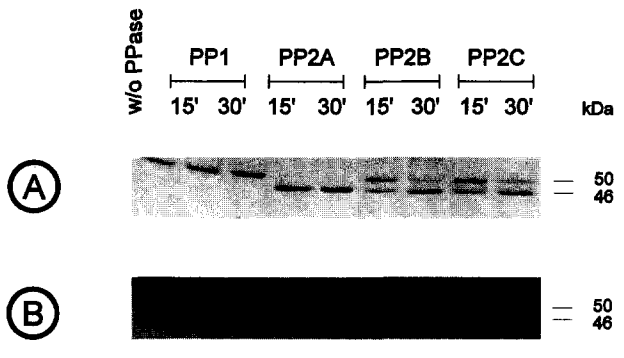

Fig. 1. Dephosphorylation of ^{32}P-phosphorylated VASP by different protein phosphatases as revealed by Coomassie blue staining (A) and autoradiography (B). Purified VASP was first phosphorylated in the presence of [γ-^{32}P]ATP using the catalytic subunit of cAK. The kinase was subsequently inhibited by PKI, and aliquots of phosphorylated VASP were then incubated with 0.5 μM of the respective phosphatase (PP1, PP2A, PP2B, PP2C) for 15 min or 30 min at 30°C. The phosphorylation state of VASP was analyzed by SDS-PAGE and autoradiography. The positions of the 46 and 50 kDa forms of VASP are indicated.

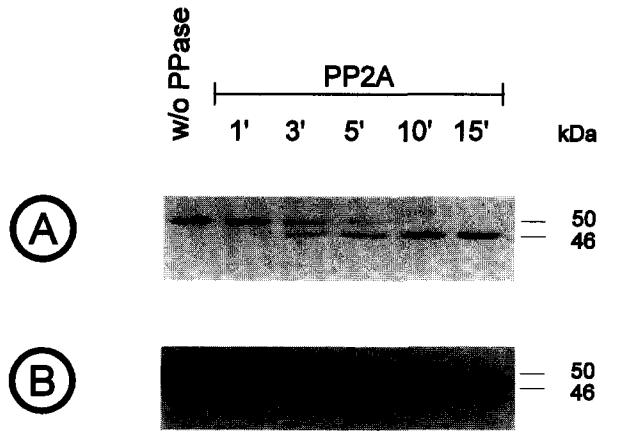

Fig. 2. Time course of VASP dephosphorylation by PP2A analyzed by Coomassie blue staining (A) and ^{32}P-autoradiography (B). After phosphorylation of purified VASP (see section 2 and Fig. 1), phosphorylated VASP was incubated with 0.3 μM PP2A at 30°C. At the time points indicated, aliquots were removed from the incubation mixture for the analysis of the VASP phosphorylation state by SDS-PAGE and ^{32}P-autoradiography. The positions of the 46 and 50 kDa forms of VASP are indicated.

3. Results

3.1. VASP is dephosphorylated by different protein serine/threonine phosphatases in vitro

When ^{32}P-labeled VASP (phosphorylated by the catalytic subunit of the cAK as described in section 2) was incubated with each of four different protein phosphatases (PP), VASP dephosphorylation was demonstrated with PP2A, PP2B and PP2C, but not with PP1 (Fig. 1). VASP dephosphorylation was detected by the shift of VASP from the 50 kDa to the 46 kDa species (analyzed by SDS-PAGE and Coomassie blue staining, Fig. 1A) and the concomitant loss of incorporated ^{32}P (detected by autoradiography, Fig. 1B). The shift between the 50 kDa/46 kDa VASP form is due to the phosphorylation/dephosphorylation of VASP at Ser^{157} [13]. Under the conditions studied (Fig. 1), PP2B and PP2C catalyzed partial VASP dephosphorylation, whereas VASP was completely dephosphorylated by PP2A but not at all by PP1.

3.2. Protein phosphatase PP2A and PP2B dephosphorylate individual VASP phosphorylation sites with different selectivity

Further investigations demonstrated significant differences between PP2A- and PP2B-catalyzed VASP dephosphorylation. Dephosphorylation of VASP by PP2A resulted in the decrease of the ^{32}P-labeled, Coomassie blue-stained 50 kDa VASP form with the concomitant appearance of the 46 kDa VASP form and the transient appearance of ^{32}P-label in the 46 kDa VASP species (Figs. 1 and 2). After a 30 min incubation with PP2A,

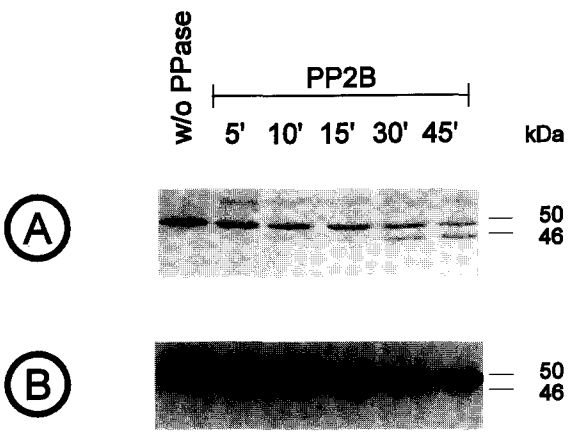

Fig. 3. Time course of VASP dephosphorylation by PP2B analyzed by Coomassie Blue staining (A) and ^{32}P-autoradiography (B). Phosphorylated VASP (see section 2 and Fig. 1) was incubated with 0.3 μM PP2B. At the time points indicated, aliquots were removed and analyzed for the phosphorylation state of VASP by SDS-PAGE and ^{32}P-autoradiography. The positions of the 46 and 50 kDa forms of VASP are indicated.

VASP had essentially lost all of its incorporated ^{32}P and was completely shifted to the 46 kDa form. A 30 min incubation of phosphorylated VASP with PP2B converted about two thirds of the 50 kDa VASP to the 46 kDa form associated with the loss of most VASP-bound radioactivity (Figs. 1 and 4). However, in contrast to the experiments with PP2A, a transient ^{32}P-labeled 46 kDa VASP form was not observed during incubations with PP2B (Figs. 1, 3, and 4). The analysis of in vitro VASP dephosphorylation by two-dimensional gel electrophoresis (Fig. 4) indicated that extensively phosphorylated VASP consists of 2–3 spots (pI range 5.4–7) with the apparent molecular mass of 50 kDa. Incubation of phosphorylated VASP with PP2B shifts VASP both to the 46 kDa form and concomitantly to more basic (46 and 50 kDa) pI values without the transient appearance of a ^{32}P-labeled 46 kDa form (Fig. 4).

3.3. *Effect of the protein phosphatase inhibitor okadaic acid on the state of VASP phosphorylation in intact washed human platelets*

Okadaic acid (OA), a potent and specific inhibitor of PP1 and PP2A in vitro and in intact cells [20], is a valuable tool for the classification and functional characterization of protein phosphatases [21]. Therefore, washed human platelets were incubated with various concentrations of OA for 40 min at 37°C, and platelet lysates were then analyzed for the state of VASP phosphorylation by two-dimensional gel electrophoresis and Western blotting. Treatment of human platelets with OA caused a concentration-dependent pI shift of VASP to more acidic values as well as a shift to the 50 kDa VASP form (Fig. 5). Both effects are already noticeable at OA concentrations as low as 50 nM (Fig. 5; other data not shown). Interestingly, the pattern of OA-induced changes in the state of VASP phosphorylation (as analyzed by 2D gel electrophoresis and Western blotting) resembled those induced by the cAMP-elevating prostacyclin analogue Iloprost and those induced by the membrane permeable cAMP analogue Sp-5,6-DCl-cBiMPS (Fig. 5). In unstimulated washed human platelets, VASP was present primarily as 46 kDa species of 2–3 spots. Although the precise pI of the most basic spot has not been determined, it approaches extremely basic values which is consistent with a pI of 9.55 calculated from the VASP amino acid sequence [14]. Iloprost, Sp-5,6-DCl-cBiMPS and OA shifted all or most of VASP to the 50 kDa form, with the extensively phosphorylated VASP comprising 2–3 spots at pI 5.4–7 (Fig. 5, Iloprost).

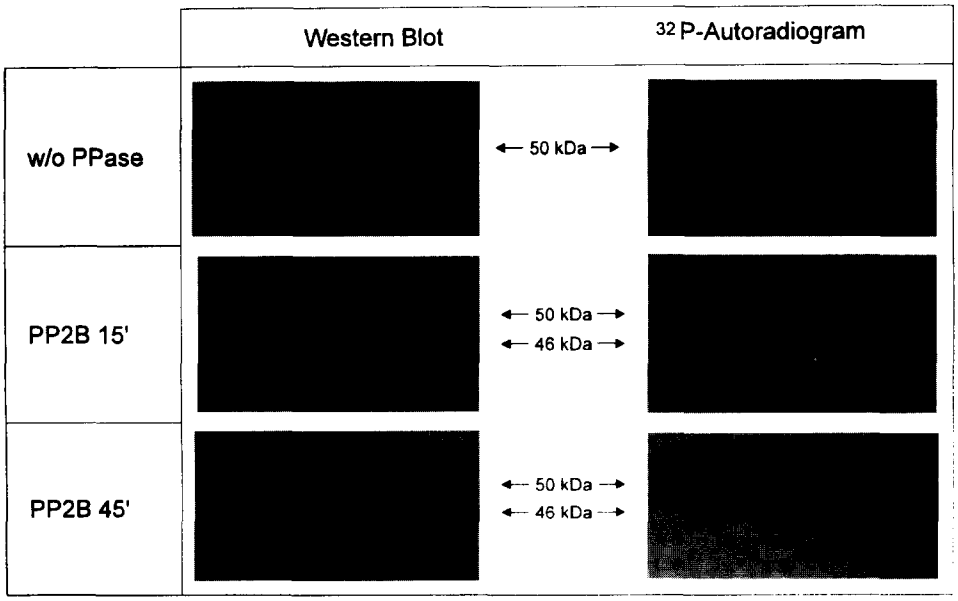

Fig. 4. Two-dimensional gel electrophoresis showing the selective dephosphorylation of VASP phosphorylation sites by PP2B. Phosphorylated VASP was incubated with PP2B for 15 min and 45 min. Aliquots of phosphorylated VASP were removed before and after incubation with PP2B and analyzed by two-dimensional gel electrophoresis followed by ^{32}P-autoradiography (right panel) and Western blotting with an ECL detection system (left panel). The positions of the 46 and 50 kDa VASP forms are indicated. The pI of internal pI standards are marked with points, from left to right pI 5.4, pI 6.8, pI 7.2.

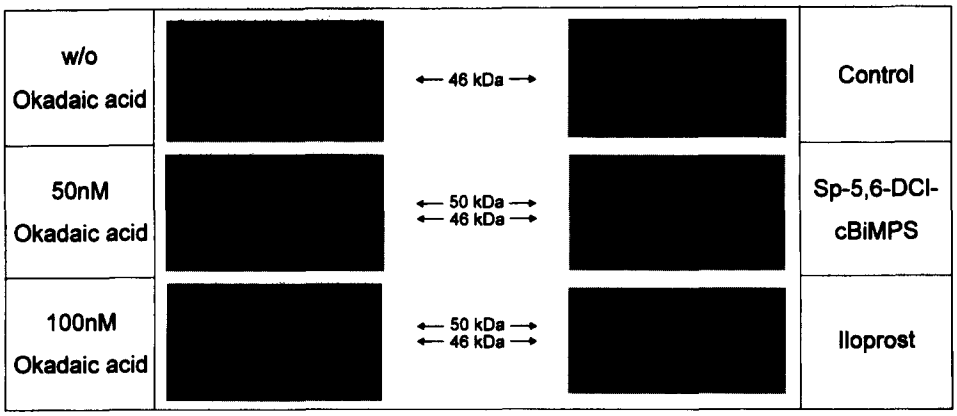

Fig. 5. Effects of okadaic acid on the VASP phosphorylation state in intact human platelets in comparison to the effects of Iloprost and Sp-5,6-DCl-cBiMPS. Washed platelets were incubated without (w/o) or with okadaic acid (50 nM or 100 nM for 40 min), or with Sp-5,6-DCl-cBiMPS (0.2 mM for 30 min) or Iloprost (5 nM for 5 min), respectively. The phosphorylation state of VASP was then analyzed by two-dimensional gel electrophoresis followed by Western blotting using anti-VASP antibodies. The positions of the 46 and 50 kDa VASP forms are indicated. The pI of internal pI standards are marked with points, from left to right pI 5.4, pI 6.8, pI 7.2.

4. Discussion

Phosphorylation of VASP, an established cAK and cGK substrate, has been extensively examined in vitro and in living cells [4–8,13]. In human platelets, reversibility of the phosphorylation-induced shift of VASP from the 46 to the 50 kDa form (due to VASP phosphorylation at Ser^{157}) has been reported to occur within a few minutes after the removal of pharmacological or physiological stimuli [8,31]. These data already suggested that protein phosphatases, in addition to protein kinases, play a major role in regulating the state of VASP phosphorylation in intact cells. However, the protein phosphatases involved have not been studied so far. Our present data demonstrate that phosphorylated VASP is a substrate of protein phosphatase PP2A, PP2B and PP2C in vitro and a substrate of an OA-sensitive phosphatase (most likely PP2A) in intact human platelets. Furthermore, the phosphatases studied dephosphorylate individual VASP phosphorylation sites with different selectivity. The transient appearance of a ^{32}P-labeled 46 kDa VASP species during PP2A-catalyzed dephosphorylation indicates that PP2A dephosphorylates the phosphorylation site responsible for the mobility shift in SDS-PAGE (Ser^{157}) somewhat faster or at least with a similar rate as compared with the dephosphorylation of the two other VASP phosphorylation sites (Ser^{239}, Thr^{278}). In contrast, the absence of a transiently labeled 46 kDa VASP species during the PP2B- and PP2C-catalyzed VASP dephosphorylation indicates that PP2B (and PP2C as well) completely dephosphorylates other phosphorylation sites (Ser^{239}, Thr^{278}) prior to dephosphorylation of Ser^{157}. The phosphatases PP1, PP2A and PP2C are known to have a broad and overlapping substrate specificity while PP2B is the most specific protein phosphatase [32,33]. The specificity of PP2B towards its substrates does not primarily depend on a specific consensus sequence but instead is mostly determined by higher-order structural features [32,34,35]. Nevertheless, in studies with phosphopeptides it has been shown [35] that an arginine at position −3 of the phosphorylation site is required for dephosphorylation by PP2B. This criterion is fulfilled by all three VASP phosphorylation sites (IERRVS^{157}NAGG, KLRKVS^{239}KQEE, RRRKAT^{278}QVGE) [14]. Furthermore, there is no acidic residue adjacent to the C-terminal side of Ser^{157} (see above) which has been characterized as a strong negative determinant. Therefore, the preferential PP2B-catalyzed dephosphorylation of VASP Ser^{239}/Thr^{278} (as compared to VASP Ser^{157}) may also be due to the higher-order structure of VASP.

Another goal of our study was the possible identification of protein phosphatase(s) involved in VASP dephosphorylation in intact cells. Incubation of intact human platelets with OA caused the accumulation of phosphorylated VASP forms. These data indicate an equilibrium between phosphorylation (protein kinases) and dephosphorylation (protein phosphatases) in resting platelets which can be shifted by phosphatase inhibitors in favor of the phosphorylated products. The OA-induced accumulation of phosphorylated VASP forms suggests OA-sensitive protein phosphatases PP1 and/or PP2A [20] as the major phosphatases responsible for VASP dephosphorylation in intact human platelets. In vitro, a distinction between PP1 and PP2A can be made by the OA concentration needed for protein phosphatase inhibition. Whereas in vitro PP2A is completely inhibited by 1 nM OA, PP1 is unaffected by this concentration [26]. However, it is difficult to distinguish PP1 and PP2A in intact cell preparations by the use of OA alone because (due to the high intracellular concentration of protein phosphatases which may range from 0.1–1 μM [26,36]) higher OA concentrations may be required. In human platelets, the major serine/threonine protein phosphatases detected are PP1, PP2A and PP2B [15,16,18]. This information, our experiments with purified protein phosphatases (Figs. 1–4) and the results obtained with OA (Fig. 5) therefore suggest that the principal protein phosphatase responsible for VASP dephosphorylation in intact human platelets is PP2A.

OA-induced accumulation of phosphorylated VASP resembled that induced by platelet cAK activators (e.g. Iloprost and Sp-5,6-DCl-cBiMPS) as shown in Fig. 5. These results together with the observation that OA preincubation [15,22–25] as well as cAK or cGK stimulation [2,3,37,38] inhibit thrombin-induced platelet activation and associated events such as aggrega-

tion, Ca^{2+} mobilization and serotonin release suggest that the inhibitory OA effects on intact human platelets are, at least in part, due to elevated phosphorylation of cAK substrates.

Repeatedly, the phosphorylation of an unidentified 50 kDa protein after platelet incubation with OA has been reported [15,23,24]. Based on the comparison of two-dimensional gel electrophoresis, effects of cAMP-elevating agents such as forskolin or prostaglandin E_1 and associated functional effects [15,23] our present data suggest that the 50 kDa unidentified phosphoprotein of these previous studies is VASP. Although VASP phosphorylation is a very sensitive marker of OA action in human platelets we certainly do not want to rule out that other phosphoproteins (including other cAK substrates) are important targets for the effects of protein phosphatase inhibitors. This appears to be especially relevant with respect to calyculin and tautomycin which, at low concentrations, selectively inhibited thrombin-induced Ca^{2+} influx rather than Ca^{2+} mobilization from intracellular stores [22].

In conclusion, our present results demonstrate that the three established VASP phosphorylation sites are selectively used not only as protein kinase substrates [13] but also as protein phosphatase substrates. In intact human platelets, VASP phosphorylation is not only increased by cAK/cGK activation but also by protein phosphatase inhibition which most likely involves PP2A. These data suggest a tight balance between VASP phosphorylation and dephosphorylation which, at least in human platelets, can be rapidly altered by the regulation of either protein kinases [29] or protein phosphatases (this study). Considering the potentially important role of VASP as link between signal transduction pathways and the regulation of cell motility [11,12] our present data should be helpful for future attempts to define the regulatory role of the various VASP phosphorylation sites in intact cells at the molecular level.

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