Stoichiometric and Reversible Phosphorylation of a 46-kDa Protein in Human Platelets in Response to cGMP- and cAMP-elevating Vasodilators*

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Recently, we reported the purification of a 46-kDa membrane-associated platelet protein which is phosphorylated in intact platelets and platelet membranes by cGMP- and cAMP-dependent protein kinases (Halbträgge, M., and Walter, U. (1989) Eur. J. Biochem. 185, 41-50). Here we demonstrate that both cGMP- and cAMP-dependent protein kinases catalyze the rapid incorporation of up to 1.4 mol of phosphate/mol of this purified vasodilator-stimulated phosphoprotein (VASP). A specific rabbit antiserum was prepared which recognized both the 46-kDa dephospho form and the 50-kDa phospho form of VASP in Western blots. In untreated washed platelets, VASP was found to be present primarily as a 46-kDa dephosphoprotein. Sodium nitroprusside (100 μM) raised the intracellular platelet cGMP concentration from ~0.44 to 4.1 μM, without a significant effect on the cAMP level, and shifted up to 67% of VASP to the 50-kDa phospho form. Prostaglandin E1 (10 μM) raised the platelet cAMP concentration from ~4.4 to 28.4 μM, without a significant effect on the cGMP level, and shifted up to 67% of VASP to the 50-kDa phospho form. Removal of the vasodilators sodium nitroprusside and prostaglandin E1 from the platelet suspension was followed by a return of the cyclic nucleotide concentration to basal levels and subsequent conversion of the 50-kDa phospho form of VASP to the 46-kDa dephospho form. The results support the hypothesis that VASP phosphorylation is an important component of the intracellular mechanism of action of these vasodilators in human platelets.

Phosphorylation Experiments with Purified VASP—Recently, our laboratory established a procedure to purify the 46-kDa vasodilator-stimulated phosphoprotein from human platelets and also demonstrated that purified VASP is a substrate for purified cGK and cAK (6). We have now investigated the stoichiometry of VASP phosphorylation by cGK and the catalytic subunit of cAK. Under the conditions used (0.96 μM VASP, 0.51 μM protein kinase, and 18 μM ATP), both cGK and the catalytic subunit of cAK rapidly phosphorylated VASP, resulting in a maximum phosphate incorporation of about 1.2–1.4 mol/mol of VASP within 15 min (Fig. 1B and Fig. 2). In several separate experiments using two different VASP preparations, the maximum phosphate incorporation was 1.3 ± 0.3 mol/mol of VASP using either cGK or cAK (Table I). This stoichiometry is based on the VASP protein concentration determined with the Bio-Rad protein assay system using catalase as standard. If bovine serum albumin was used as standard in this protein assay system.

EXPERIMENTAL PROCEDURES*  

RESULTS

Phosphorylation Experiments with Purified VASP—Recently, our laboratory established a procedure to purify the 46-kDa vasodilator-stimulated phosphoprotein from human platelets and also demonstrated that purified VASP is a substrate for purified cGK and cAK (6). We have now investigated the stoichiometry of VASP phosphorylation by cGK and the catalytic subunit of cAK. Under the conditions used (0.96 μM VASP, 0.51 μM protein kinase, and 18 μM ATP), both cGK and the catalytic subunit of cAK rapidly phosphorylated VASP, resulting in a maximum phosphate incorporation of about 1.2–1.4 mol/mol of VASP within 15 min (Fig. 1B and Fig. 2). In several separate experiments using two different VASP preparations, the maximum phosphate incorporation was 1.3 ± 0.3 mol/mol of VASP using either cGK or cAK (Table I). This stoichiometry is based on the VASP protein concentration determined with the Bio-Rad protein assay system using catalase as standard. If bovine serum albumin was used as standard in this protein assay system.

* Portions of this paper (including “Experimental Procedures,” Figs. 2, 5, and 7-10, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
VASP (Fig. 1C). Both Coomassie Blue staining (Fig. 1A) and radioimmunolabeling (A), autoradiography (B), and radioimmunolabeling (C). Purified VASP (6.2 μg, 0.96 μM) was phosphorylated as described under “Experimental Procedures” for the times indicated using 0.51 μM cGK (upper panels) or a 0.51 μM concentration of the catalytic subunit (C) of cAK (lower panels). Proteins were separated by SDS-PAGE and detected by Coomassie Blue staining (A), autoradiography of the phosphorylated proteins (B), and radioimmunolabeling (C) using an antiserum against VASP. In A and B, each assay point corresponds to the equivalent of 596 ng of VASP, whereas 100 ng of VASP/lane was analyzed by Western blot in C. Molecular masses of protein standards (PS) are indicated in A.

VASP protein concentration was 2.5-fold lower, and the specific phosphate incorporation was consequently 2.5-fold higher than that determined with catalase as standard. However, the data obtained with catalase as standard are used here since the color reaction of catalase with the dye reagent better represents the majority of proteins (see Ref. 21).

Phosphorylation of VASP by cGK or cAK also resulted in a shift of the apparent molecular mass of VASP on SDS-PAGE from 46 to 50 kDa (Figs. 1, A and B). Transient phosphorylation of the 46-kDa species of VASP was observed with only cGK, but not cAK (Fig. 1B).

The shift in the apparent molecular mass of VASP after phosphorylation was also detected by a radioimmunolabeling (Western blot) method using a rabbit antiserum against VASP (Fig. 1C). Both Coomassie Blue staining (Fig. 1A) and radioimmunolabeling (Fig. 1C) also demonstrated that VASP was purified predominantly as a 46-kDa protein and that it could be completely converted to the 50-kDa protein by cGK- or cAK-catalyzed phosphorylation.

 Regulation of VASP Phosphorylation by Cyclic Nucleotide-

Fig. 1. Time course of purified VASP phosphorylation by cGK and catalytic subunit of cAK shown by Coomassie Blue staining (A), autoradiography (B), and radioimmunolabeling (C). Purified VASP (6.2 μg, 0.96 μM) was phosphorylated as described under “Experimental Procedures” for the times indicated using 0.51 μM cGK (upper panels) or a 0.51 μM concentration of the catalytic subunit (C) of cAK (lower panels). Proteins were separated by SDS-PAGE and detected by Coomassie Blue staining (A), autoradiography of the phosphorylated proteins (B), and radioimmunolabeling (C) using an antiserum against VASP. In A and B, each assay point corresponds to the equivalent of 596 ng of VASP, whereas 100 ng of VASP/lane was analyzed by Western blot in C. Molecular masses of protein standards (PS) are indicated in A.

Fig. 3. Autoradiograph showing time course of VASP phosphorylation in intact platelets analyzed by Western blot. Washed human platelets were incubated with 100 μM SNP (A) or 10 μM PGE1 (B) for the times indicated. The incubation was terminated, proteins were separated on a 9% SDS-polyacrylamide gel, and VASP phosphorylation was assayed by Western blot as described under “Experimental Procedures.”
had no significant effects on the platelet cAMP level (Figs. 4 and 5).

Although SNP effects on platelet cGMP levels and VASP phosphorylation did not appear to reach plateau levels with the SNP concentrations studied, it is of interest that small effects on cGMP levels and VASP phosphorylation were already detectable with 0.1–1.0 μM SNP (Fig. 5). PGE1 (10 μM) converted up to 60% of VASP to the 50-kDa protein within 1–2 min, and this conversion was preceded by a rapid 6.5-fold increase in platelet cAMP content from 21.8 (corresponding to 4.4 μM) to 142 (corresponding to 28.4 μM) pmol/10⁹ cells, as demonstrated in Fig. 6. PGE1 had very little, if any, effect on platelet cGMP content (Figs. 6 and 7). Effects on platelet cAMP content and VASP phosphorylation were detectable with 0.01–0.1 μM PGE1, and were maximal with 1.0–10.0 μM PGE1 (Fig. 7). Platelet cyclic nucleotide content and the level of the 50-kDa protein of VASP eventually slowly declined despite the continued presence of SNP or PGE1 (Figs. 4 and 6).

Washed human platelets incubated with cell membrane-permeable cyclic nucleotide analogs, 1 mM 8-chlorophenylthio-cAMP (8-CPT-cAMP) and 1 mM 8-bromo-cGMP (8-Br-cGMP), showed a 70 and 45% conversion of VASP to the 50-kDa protein, respectively (Fig. 8). The 8-Br-cGMP effect had a slower onset than that of 8-CPT-cAMP.

Reversibility of Effects of Vasodilators on Platelet Cyclic Nucleotide Content and VASP Phosphorylation—Removal of SNP from the platelet suspension by washing resulted in a rapid decline of the elevated cGMP content to near basal levels, followed by a reverse conversion of VASP from the 50-kDa protein to the 46-kDa protein, as shown in Fig. 9. Similarly, removal of PGE1 from the platelet suspension caused the return of the elevated cAMP content to basal levels, followed by a complete reverse conversion of VASP from the 50-kDa protein to the 46-kDa protein (Fig. 10).

Inhibition of Platelet Aggregation by SNP, PGE1, and Cyclic Nucleotide Analogs—In order to search for a possible correlation between vasodilator-stimulated phosphorylation of the 46-kDa protein and vasodilator-induced inhibition of platelet aggregation, the effect of SNP, PGE1, 8-Br-cGMP, and 8-CPT-cAMP on the aggregation of washed human platelets was studied. Although the results varied substantially between platelet preparations prepared from different donors, the threshold concentrations causing aggregation inhibition were usually 0.1–1.0 μM SNP, 0.01–0.1 μM PGE1, 50–100 μM 8-Br-cGMP, and 25–50 μM 8-CPT-cAMP, whereas complete inhibition of aggregation was observed with 10–100 μM SNP, 1–10 μM PGE1, 0.5–1.0 μM 8-Br-cGMP, and 0.25–0.5 mM 8-CPT-cAMP (data not shown). These results were obtained using a preincubation time of 5 min with SNP or PGE1 and of 10 min with 8-Br-cGMP or 8-CPT-cAMP.
DISCUSSION

Recent purification (5) of a 46-kDa platelet protein (referred to as VASP) previously shown (4) to be phosphorylated in response to cGMP- and cAMP-elevating vasodilators made possible this study of certain kinetic and stoichiometric aspects of the phosphorylation of this protein in either its purified or endogenous state in intact human platelets. Both the catalytic subunit of cAK and cGK rapidly phosphorylated VASP, resulting in a maximum phosphate incorporation of 1.2–1.4 mol/mol of VASP (Fig. 1 and Table 1). This phosphorylation altered the mobility of VASP on SDS-PAGE, shifting its apparent molecular mass from 46 to 50 kDa. We interpret these phosphate incorporation results and the finding that transient phosphorylation of the 46-kDa species of VASP is observed with cGK, but not with cAK (Fig. 1), as evidence for the presence of two distinct phosphorylation sites in VASP. These two distinct sites are phosphorylated by both cAK and cGK, but cAK appears to phosphorylate the site responsible for the shift in apparent molecular mass more efficiently than the second site since transient phosphorylation of the 46-kDa species of VASP is absent or negligible with cAK. In contrast, cGK appears to phosphorylate these two sites with similar efficiency since transient phosphorylation of the 46-kDa species of VASP is observed. Other data published previously (4, 5) suggest that the two distinct phosphorylation sites are both serine residues located in close proximity since tryptic fingerprinting and phosphoamino acid analysis of the 46- and 50-kDa species of VASP phosphorylated by either cAK or cGK only produced one major phosphopeptide and only phosphoserines. However, this point will have to be confirmed by microsequencing of the phosphorylation sites.

Under our in vitro phosphorylation conditions (0.96 µM VASP, 0.51 µM cGK or cAK, and 18 µM ATP), much lower concentrations of protein kinases and substrates were used than those present in intact human platelets. Recent studies3 using specific antisera against cGK, the catalytic subunit of cAK, and VASP suggest an intracellular protein concentration of 3.5 µM catalytic subunit of cAK (assuming a molecular mass of 40 kDa), 6 µM cGK (assuming a molecular mass of 46 kDa), and 8–16 µM VASP (assuming a molecular mass of 46 kDa), whereas the intracellular concentration of ATP is generally assumed to be in the millimolar range. The results indicating rapid and stoichiometric phosphorylation of VASP in vitro (Fig. 2) and the very high concentrations of protein kinases and their substrates in intact platelets suggest that intact human platelets have the capacity for a rapid and stoichiometric phosphorylation of VASP via activation of either cGK or cAK. To test this with intact platelets, an antiserum was prepared which recognized both the phospho (50 kDa) and dephospho (46 kDa) forms of VASP (Fig. 1C).

The properties of this antiserum made possible an analysis of the state of phosphorylation of VASP by the Western blot method. A similar method has been used to study the phosphorylation of type II regulatory subunit of cAK in intact cells (8).

Initial studies demonstrated that purified VASP and VASP of untreated washed platelets consisted primarily of the 46-kDa protein (Figs. 1 and 3). For practical reasons, we use the term 50-kDa protein interchangeably with phospho-VASP and the term 46-kDa protein interchangeably with dephospho-VASP. However, the Western blot method used is not capable of distinguishing nonphosphorylated 46-kDa VASP from 46-kDa VASP transiently phosphorylated by cGK.

Therefore, our study of VASP phosphorylation in intact platelets principally analyzes the phosphorylation/dephosphorylation of the site which is responsible for the shift in apparent molecular mass of VASP. SNP and PGE1 were capable of producing a 65–70% conversion of VASP to the 50-kDa phospho form, a conversion which was preceded by an elevation of the intracellular concentration of either cGMP or cAMP, respectively (Figs. 4–7). Since SNP and PGE1 had no significant effects on the level of cAMP or cGMP, respectively, the phosphorylation of VASP induced by SNP appears to occur via activation of cGMP, and the phosphorylation induced by PGE1 via activation of cAK.

It is of interest to note that whereas the intracellular concentration of cGMP under basal conditions (~0.44 µM) is more than an order of magnitude lower than that of the cGMP-binding sites of cGK (~12 µM, assuming two cGMP-binding sites/cGK monomer), the basal cAMP concentration (~4.4 µM) is of the same order of magnitude as the cAMP-binding site concentration of cAK (~7 µM, assuming two cAMP-binding sites/regulatory subunit and equimolar concentrations of the regulatory and catalytic subunits). This may explain the observation that the onset of VASP phosphorylation induced by SNP or 8-Br-cGMP is slower than that of VASP phosphorylation induced by PGE1 or 8-Br-cAMP (Figs. 4, 6, and 8). Presumably, a sufficient intracellular concentration of cGMP or a cAMP analog has to accumulate in order to occupy at least some of the available free cGMP-binding sites on intracellular cGMP-binding proteins (cGK and cGMP-regulated phosphodiesterases) before activation of cGK is observed. In contrast, relatively small changes in the cAMP level may already be sufficient for full activation of cAK. Therefore, it should also be considered that SNP may cause VASP phosphorylation in intact human platelets via activation of cAK. SNP-increased cGMP could inactivate cAMP-inhibited cAMP phosphodiesterase (the major low Km cAMP phosphodiesterase in human platelets (10)), leading to elevated cAMP levels and activation of cAK. However, we consider this possibility unlikely in our experiments since 1) SNP had no significant effects on platelet cAMP concentration (Figs. 4 and 5), and 2) the pattern of protein phosphorylation induced by SNP or 8-Br-cGMP in intact human platelets was quite different than that induced by PGE1 or cAMP analogs (4). The considerably lower lipophilic properties of 8-bromo derivatives of cyclic nucleotides, compared to 8-chloro-9-(4-thio)adenosine-1,3,5(9)-triphosphate, the major low Km cAMP phosphodiesterase in human platelets (10), leading to elevated cAMP levels and activation of cAK.

Removal of SNP or PGE1 from the platelet suspension resulted in a prompt return of the cGMP or cAMP concentration, respectively, to near basal levels, followed by a conversion of 50-kDa phospho-VASP to 46-kDa dephospho-VASP (Figs. 9 and 10). These results demonstrate that intact human platelets have not only a significant capacity for cyclic nucleotide synthesis and cyclic nucleotide-dependent protein phosphorylation, but also powerful activities catalyzing the degradation of cGMP and cAMP and the dephosphorylation of proteins such as VASP.

In conclusion, we interpret these data as evidence for near stoichiometric and reversible phosphorylation of VASP which occurs in intact human platelets in response to cyclic nucleotide-elevating vasodilators via activation of either cGK or cAK. The reasonably good correlation between the concentra-

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tion- and time-dependent effects of cyclic nucleotide-elevating vasodilators on platelet aggregation and VASP phosphorylation suggests that VASP phosphorylation may be a component important for the inhibitory effects of these vasodilators on platelet activation. This is also supported by the findings that cAMP- and cGMP-elevating vasodilators have synergistic effects with respect to both the inhibition of platelet activation (12, 13) and VASP phosphorylation. However, more than one pathway for the inhibition of platelet activation may exist (3). Vasodilators also appear to regulate the phosphorylation of other platelet proteins such as glycoprotein Ibα (14), cGMP-inhibited cAMP phosphodiesterase (15), and a ras-related GTP-binding protein (16, 17) via activation of cAK. The precise mechanism for the inhibition of platelet aggregation by cyclic nucleotide-elevating vasodilators has not been determined, although it appears to occur at an early step of the activation cascade, possibly at the level of phospholipase C (1, 3, 18-20). We hope that our ongoing biochemical and cell biological studies with human platelets will elucidate the function of cAMP- and cGMP-mediated phosphorylation of VASP and contribute to the understanding of the mechanism of action of cyclic nucleotide-elevating vasodilators.

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FIG. 2. Time course of purified VASP phosphorylation by cGK and catalytic subunit of cGK. Purified VASP [35S] was phosphorylated by cGK or catalytic subunit of cGK for various times and separated on a 5% SDS-polyacrylamide gel as shown in Figure 1A and B. VASP phosphorylation by VASP was determined by varying time and varying CPM for each time. Data from this figure were representative for four separate experiments using two different preparations of VASP.

Sodium Nitroprusside

Prostaglandin E1

8-Bromo-cGMP

8-CPT-cAMP

FIG. 5. Concentration-dependent effects of SNP on VASP phosphorylation (A) and cyclic nucleotides (B) in intact platelets. Washed human platelets were incubated with the indicated concentrations of SNP for 1 minute. Amounts of the 68 kDa protein (dephospho-VASP; △△, 60 kDa protein (phospho-VASP; ◊◊◊)) were determined as described under "Experimental Procedures." Data shown represent means ± SD of 3 separate experiments.

FIG. 7. Concentration-dependent effects of PG-E1 on VASP phosphorylation (A) and cyclic nucleotides (B) in intact platelets. Washed human platelets were incubated with the indicated concentrations of PG-E1 for 1 minute. Amounts of the 68 kDa protein (dephospho-VASP; △△, 60 kDa protein (phospho-VASP; ◊◊◊)) were determined as described under "Experimental Procedures." Data shown represent means ± SD of 3 separate experiments.

FIG. 9. Reversibility of the effects of SNP on VASP phosphorylation (A) and the cyclic nucleotides (B). Washed human platelets were incubated with the indicated concentrations of SNP for 1 minute. Amounts of the 68 kDa protein (dephospho-VASP; △△, 60 kDa protein (phospho-VASP; ◊◊◊)) were determined as described under "Experimental Procedures." Data shown represent means ± SD of 3 separate experiments.
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