Pergamon

*Biochemical Pharmacology*, Vol. 48, No. 8, pp. 1569–1575, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00340-8

# SYNERGISTIC PHOSPHORYLATION OF THE FOCAL ADHESION-ASSOCIATED VASODILATOR-STIMULATED PHOSPHOPROTEIN IN INTACT HUMAN PLATELETS IN RESPONSE TO cGMP- AND cAMP-ELEVATING PLATELET INHIBITORS

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(*Received* 2 *May* 1994; *accepted* 26 *July* 1994)

**Abstract**—The mechanism underlying the synergistic inhibition of platelet activation by cGMP- and cAMP-elevating vasodilators was investigated using washed human platelets and platelet-rich plasma. With both types of human platelet preparations, low concentrations of sodium nitroprusside increased the cAMP-elevating potency of low concentrations of prostaglandin $E_1$ (PG-$E_1$). Using threshold concentrations of both sodium nitroprusside and PG-$E_1$, the NO-donor potentiated the effect of PG-$E_1$ with respect to the phosphorylation of the focal adhesion-associated vasodilator-stimulated phosphoprotein (VASP) at serine$^{157}$. In contrast, threshold concentrations of cell-membrane permeant selective activators of the platelet cGMP-dependent protein kinase or the cAMP-dependent protein kinase had only additive effects on VASP serine$^{157}$ phosphorylation in washed human platelets. The data demonstrate that low intracellular levels of cGMP effectively inhibit type III cGMP-inhibited phosphodiesterase in human platelets despite the high levels of cGMP-dependent protein kinase present in this cell type. This study provides the first evidence that the simultaneous activation of both cGMP- and cAMP-dependent protein kinase results in additive effects on VASP serine$^{157}$ phosphorylation, whereas the supra-additive effects observed with the combination of sodium nitroprusside and PG-$E_1$ are due to cGMP-mediated inhibition of type III phosphodiesterase. VASP phosphorylation at serine$^{157}$ may be an important component underlying the synergistic inhibition of human platelets by cGMP- and cAMP-elevating agents.

*Key words:* nitric oxide; prostaglandins; cyclic nucleotides; protein kinases; synergism

The activation of platelets is tightly regulated under physiological conditions and often pathologically increased in diseases such as thrombosis, atherosclerosis, diabetes and hypertension [1]. Numerous vasoactive substances including hormones, autacoids and drugs synergistically stimulate or inhibit the activation of platelets [2]. The interaction between intracellular $Ca^{2+}$ and protein kinase C appears to be a major factor in determining the synergistic activation of human platelets observed with low concentrations of agents such as thrombin, collagen, ADP, thromboxane $A_2$, vasopressin, adrenaline and platelet activating factor [3, 4]. It is also well established that platelet activation is strongly inhibited by agents which elevate either cAMP or cGMP [1, 2, 5]. Moreover, low concentrations of both physiological and pharmacological cAMP- and cGMP-elevating agents synergistically inhibit platelet activation *in vitro* [6–9] and *in vivo* [10, 11]. However, the molecular basis of the synergistic inhibition of platelet function by cGMP- and cAMP-elevating agents is not well understood. In most cells including human platelets, the intracellular effects of cAMP are primarily mediated by cAMP-dependent protein kinases (cA-PK†) and their substrates [5]. In contrast, cGMP may achieve its intracellular effects by more than one mechanism, including regulation of cGMP-dependent protein kinases (cG-PK), cyclic nucleotide PDE and cGMP-gated cation channels [5, 12]. Considerable evidence suggests that stimulation of either cA-PK or cG-PK in human platelets inhibits agonist-evoked calcium mobilization and subsequent $Ca^{2+}$-/protein kinase C-mediated protein phosphorylation [5, 13–15]. Experiments with membrane-permeant selective activators of the cG-PK and with cG-PK deficient human platelets demonstrated that the platelet cG-PK is an important mediator of the effects of cGMP-elevating platelet inhibitors [13, 14]. However, cGMP-elevating agents also increase the cAMP-elevating capacity of adenylyl cyclase activators, an effect mediated by the inhibition of type III cGI-

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† Abbreviations: cA-PK, cAMP-dependent protein kinase; cG-PK, cGMP-dependent protein kinase; PDE, phosphodiesterase; cGI-PDE, cGMP-inhibited PDE (type III); VASP, vasodilator-stimulated phosphoprotein; PG-$E_1$, prostaglandin $E_1$; SNP, sodium nitroprusside; Sp-5,6-DCl-cBiMPS, Sp-5,6-dichloro-1-$\beta$-D-ribofuranosyl-benzimidazole-3′,5′-monophosphorothioate; 8-pCPT-cGMP, 8-(*p*-chlorophenylthio)-cGMP; PRP, platelet-rich plasma.

PDE by cGMP as clearly demonstrated with rabbit platelets [16, 17]. Our laboratory purified a 46 kDa platelet protein termed VASP and demonstrated the stoichiometric phosphorylation of VASP by both cA-PK and cG-PK in intact human platelets which closely correlated with the inhibition of platelet activation [18–20]. More recently, VASP was characterized as a novel focal adhesion protein [21] which contains three phosphorylation sites used by the cA-PK and cG-PK *in vitro* and in intact cells [22]. One of these three sites (serine$^{157}$) is efficiently used by both cA-PK and cG-PK in intact cells and is responsible for the well established phosphorylation-induced shift of VASP from the 46 to the 50 kDa form in SDS–PAGE [18, 20, 22]. Together with the measurement of cyclic nucleotides, the analysis of the phosphorylation-induced shift of VASP from the 46 to the 50 kDa form can be used to quantitatively assess the cA-PK- or cG-PK-mediated VASP phosphorylation in intact human platelets and other cells [19–22]. These methods were used to demonstrate the endothelium-dependent phosphorylation of platelet VASP using a reconstituted cell culture system [23] or the intact coronary vascular bed [24]. In the present study, these methods were used to quantitatively measure the possible synergistic effects of cGMP- and cAMP-elevating platelet inhibitors on cG-PK- and cA-PK-mediated protein phosphorylation in intact human platelets. The results obtained suggest that the synergistic VASP phosphorylation observed in response to the two types of platelet inhibitors consists of two components, an additive component due to the activation of both cG-PK and cA-PK and a supra-additive component due to the cGMP-mediated inhibition of type III PDE.

## MATERIALS AND METHODS

$^{125}$I-Protein A (30 mCi/mg) and the $^{125}$I-cAMP assay system were obtained from Amersham Buchler (Braunschweig, Germany), $^{125}$I-cGMP from Dupont (Bad Homburg, Germany). PG-$E_1$ and SNP were purchased from Sigma (Munich, Germany) and diluted as described [25]. Sp-5,6-DCI-cBiMPS and 8-pCPT-cGMP were obtained from Biolog (Bremen,

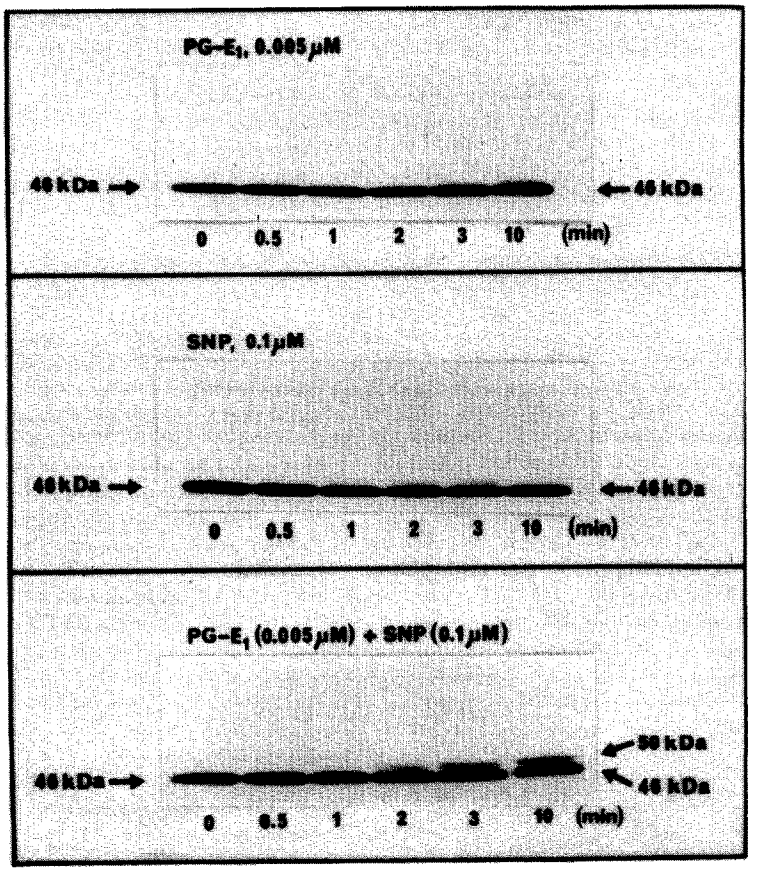


Fig. 1. Autoradiographs showing the time course of VASP phosphorylation in intact human platelets in response to PG-$E_1$, SNP or the combination of SNP and PG-$E_1$. Washed human platelets ($1 \times 10^9$ cells/mL) were incubated with 0.005 µM PG-$E_1$ (upper panel), 0.1 µM SNP (middle panel) or with both substances (lower panel). Aliquots ($0.6 \times 10^8$ platelets) were removed from the suspension at the time points indicated and mixed with an SDS-containing stop solution. Proteins were separated on SDS–PAGE and VASP phosphorylation was analysed by western blots. The position of the 46 and 50 kDa forms of VASP is indicated.

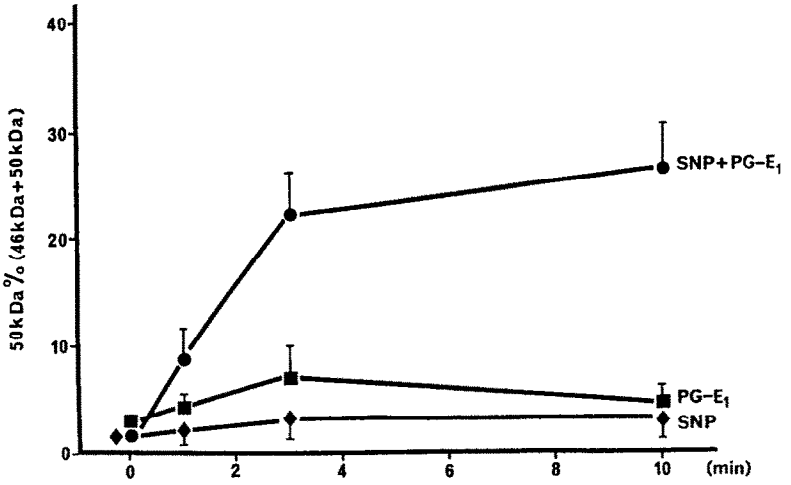


Fig. 2. Time course of the effects of SNP, PG-$E_1$ or the combination of SNP and PG-$E_1$ on VASP phosphorylation in intact human platelets. Washed human platelets were incubated with 0.1 $\mu$M SNP, 0.005 $\mu$M PG-$E_1$ or with the combination of 0.1 $\mu$M SNP and 0.005 $\mu$M PG-$E_1$. Aliquots ($0.6 \times 10^8$ cells) were withdrawn at the time points indicated and mixed with an SDS-containing stop solution. VASP phosphorylation was analysed by western blots and is expressed as appearance of the 50 kDa VASP as percentage of total VASP (46 + 50 kDa forms). Data shown represent the means (± SEM) of four separate experiments.

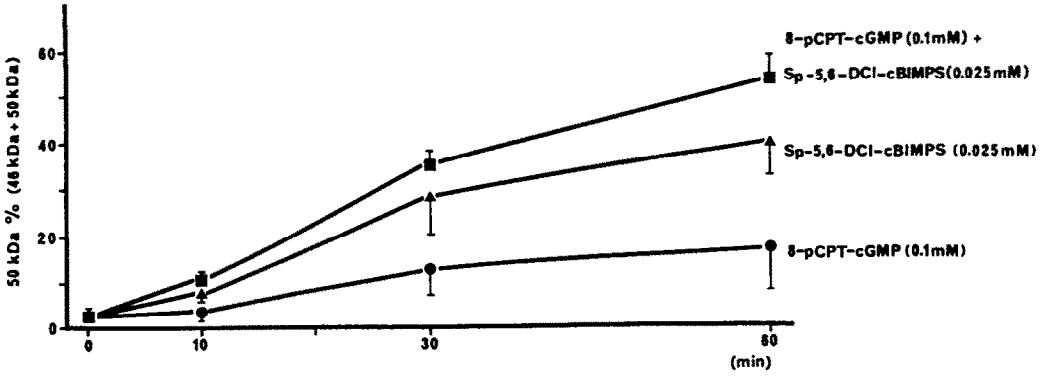


Fig. 3. Time course of the effects of 8-pCPT-cGMP, Sp-5,6-DCl-cBiMPS or the combination of both cyclic nucleotide analogs on VASP phosphorylation in intact human platelets. Washed human platelets were incubated with 0.1 mM 8-pCPT-cGMP, 0.025 mM Sp-5,6-DCl-cBiMPS or with the combination of both substances. Aliquots ($0.6 \times 10^8$ platelets) were taken at the time points indicated and analysed for VASP phosphorylation by western blots. The results represent the means (± SEM) of three separate experiments.

Germany). Purification of VASP from human platelets and preparation of an antiserum against VASP have been described [18, 19].

*Analysis of cyclic nucleotide levels and VASP phosphorylation in intact washed human platelets.* Isolation of washed human platelets was performed as described previously [25]. VASP phosphorylation and cyclic nucleotide content were analysed by western blots and radioimmunoassay as described previously [19, 20].

*Analysis of cyclic nucleotide levels and VASP phosphorylation in intact human platelets of PRP.* Blood from healthy donors was obtained and prepared as described previously [20]. Isolation of PRP and stimulation of PRP platelets was carried out as described in detail elsewhere [14]. Briefly, PRP was incubated with various concentrations of SNP and/or PG-$E_1$ for 5 min as indicated and then centrifuged for 10 sec at 8160 *g* and room temperature. The supernatant was immediately removed and discarded. The platelet pellet was immediately solubilized in an SDS-containing stop solution (for the analysis of VASP phosphorylation by western blots) or in 10% trichloroacetic acid (for the analysis of cyclic nucleotides) as described [14].

*Statistics.* When indicated, results are expressed as means ± SEM for (N) separate experiments, each performed with blood from different donors. Student's unpaired *t*-test was used to determine the significance of differences between means,

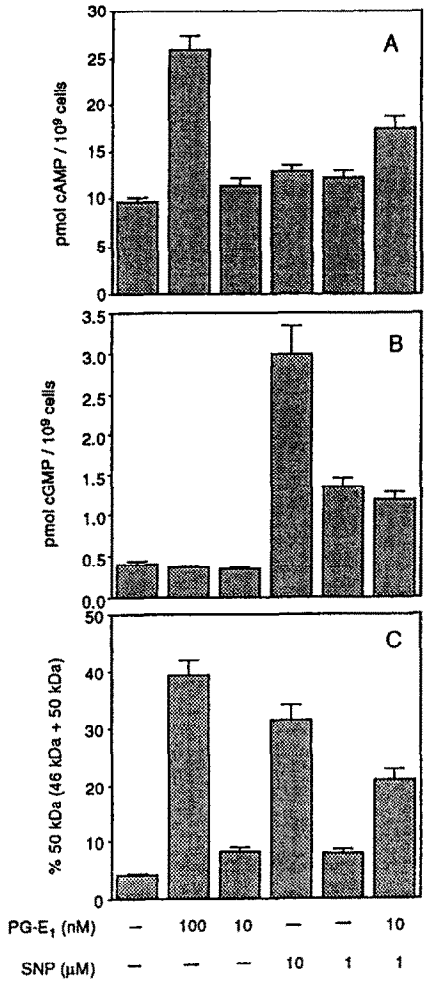


Fig. 4. Effects of PG-$E_1$, SNP or the combination of PG-$E_1$ and SNP on VASP phosphorylation and cyclic nucleotide content in platelets of PRP. PRP was incubated for 5 min without additions (−), with 100 nM or 10 nM PG-$E_1$, with 10 μM or 1 μM SNP or with the combination of 10 nM PG-$E_1$ and 1 μM SNP. Platelets were rapidly pelleted, and their content of 50 kDa VASP and cyclic nucleotides was determined by western blots and radioimmunoassay. Data shown represent the means (± SEM) of 19 separate experiments.

and a P value of <0.05 was taken as statistically significant.

## RESULTS

The extent of VASP phosphorylation in intact washed human platelets in response to low concentrations of PG-$E_1$ and SNP used alone or in combination is shown in Figs 1 and 2. Phosphorylation was quantitatively assessed by measuring the shift from the 46 to the 50 kDa VASP form due to the phosphorylation of VASP at serine[157] [22]. Therefore, all phosphorylation data reported here indicate VASP serine[157] phosphorylation. Platelets incubated with 0.005 μM PG-$E_1$ or 0.1 μM SNP alone showed very little VASP phosphorylation within 10 to 60 min (Figs 1 and 2; other data not shown). However, addition of both substances (0.005 μM PG-$E_1$ + 0.1 μM SNP) to platelets stimulated VASP serine[157] phosphorylation several-fold to approx. 22% within 3 min and 27% within 10 min (Figs 1 and 2). Additional analysis of platelet cyclic nucleotide levels confirmed earlier studies [16] that nitric oxide donors such as SNP increase the cAMP-elevating potency of PG-$E_1$ (data not shown).

For the analysis of the possible contribution of PDEs and protein kinases to the extent of VASP serine[157] phosphorylation, platelets were also incubated with Sp-5,6-DCl-cBiMPS and 8-pCPT-cGMP, which have been established as selective, hydrolysis-resistant activators of the platelet cA-PK and cG-PK, respectively [26, 27]. The time course of platelet VASP phosphorylation in response to these two cyclic nucleotide analogs used alone or in combination is shown in Fig. 3. After a 30 min incubation (data for the 60 min incubation period in parentheses), the extent of VASP phosphorylation was 14% (17%) with 0.1 mM 8-pCPT-cGMP, 28% (38%) with 0.025 Sp-5,6-DCl-cBiMPS and 37% (54%) with the two reagents used in combination (Fig. 3).

The interaction of SNP and PG-$E_1$ at the level of platelet protein phosphorylation was also investigated using platelet-rich plasma since the biological effects and stability of nitric oxide donors and prostaglandins may be different in a physiological medium such as plasma when compared to the buffers used for the preparation and resuspension of washed platelets. In addition, even the most careful procedures used for the preparation of washed human platelets may alter the biological response of these cells. Incubation of PRP with relatively high concentrations of PG-$E_1$ (100 nM) or SNP (10 μM) alone caused extensive VASP serine[157] phosphorylation associated with the expected cAMP or cGMP increase, respectively (Fig. 4). As indicated in the legend of Fig. 4, all data obtained with PRP experiments represent the means (±SEM) of 19 separate experiments. PG-$E_1$ (10 nM) had small, but significant effects on platelet cAMP levels (increase from 9.6 ± 0.5 to 11.3 ± 0.7 pmol/$10^9$ cells; P = 0.030) and VASP phosphorylation (increase from 4.2 ± 0.4 to 8.1 ± 0.9%; P < 0.00005). SNP (1 μM) significantly increased platelet cGMP levels (from 0.4 ± 0.05 to 1.3 ± 0.12 pmol/$10^9$ cells, and VASP phosphorylation (from 4.2 ± 0.4 to 7.9 ± 0.7%; P < 0.00005) associated with a small, but significant effect on cAMP levels (increase from 9.6 ± 0.5 to 12.1 ± 0.8 pmol/$10^9$ cells; P = 0.0014). When 10 nM PG-$E_1$ and 1 μM SNP were used in combination, substantial VASP serine[157] phosphorylation was clearly observed (Fig. 4). The increased VASP serine[157] phosphorylation observed in response to the combination of 10 nM PG-$E_1$ + 1 μM SNP (20.9–4.2% background phosphorylation = 16.7% increased phosphorylation) was significantly more (P = 0.00007) than the expected sum (7.6%) of the increased VASP serine[157] phosphorylation measured in response to 10 nM PG-$E_1$ (3.9%) or 1 μM SNP (3.7%) alone. Cyclic nucleotide analysis of platelets incubated with both 10 nM PG-$E_1$ and 1 μM SNP demonstrated a substantial increase (when compared to control platelets) of cAMP (rise from 9.6 ± 0.5

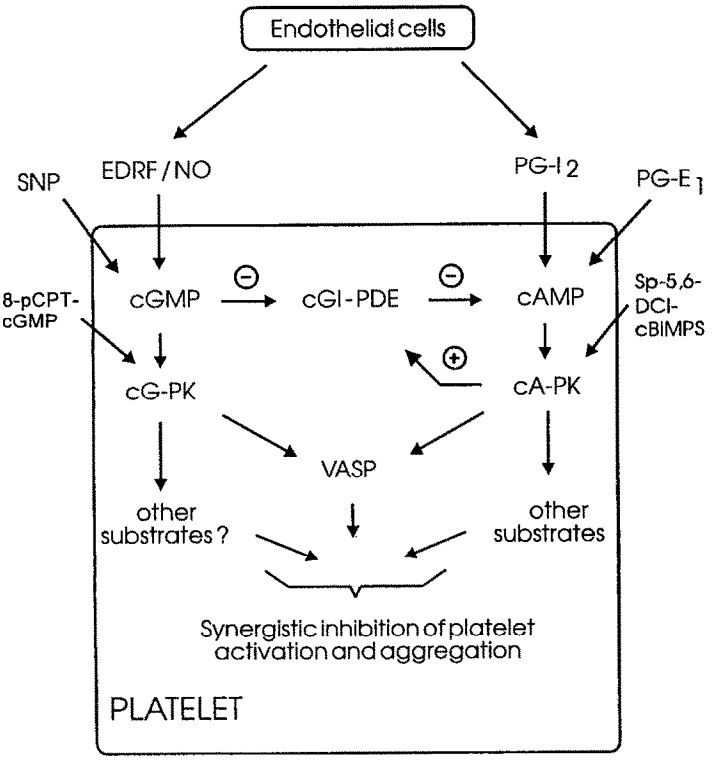


Fig. 5. Model showing the underlying mechanisms for the synergistic effects of cyclic nucleotide-elevating agents on VASP phosphorylation and platelet function. cGMP-elevating agents (i.e. SNP, EDRF/NO) and cAMP-elevating prostaglandins (i.e. PG-$E_1$, PG-$I_2$) used alone stimulate VASP phosphorylation in human platelets mediated by the cG-PK or cA-PK, respectively. When used in combination, the effects of cAMP-elevating agents on VASP phosphorylation and platelet function are additionally amplified by cGMP-elevating agents due to the inhibitory effects of cGMP on cGI-PDE type III. Direct activation of cG-PK and cA-PK by kinase-selective membrane-permeant cyclic nucleotide analogs (8-pCPT-cGMP, Sp-5,6-DCl-cBiMPS) results in additive effects on platelet VASP phosphorylation.

to 17.4 ± 1.3 pmol/$10^9$ cells) and cGMP (rise from 0.4 ± 0.05 to 1.2 ± 0.1 pmol/$10^9$ cells). PG-$E_1$ used with or without SNP did not significantly affect platelet cGMP levels (Fig. 4).

## DISCUSSION

Previous investigations documented the synergistic inhibitory effects of cGMP- and cAMP-elevating agents on platelet activation *in vitro* and *in vivo* [6–11]. Using rabbit platelets, Maurice and Haslam demonstrated that nitrovasodilator-caused, cGMP-mediated inhibition of type III cGI-PDE increased the cAMP-elevating potency of adenylyl cyclase activators, which appears to be an important component underlying the synergistic effect of cyclic nucleotide-elevating platelet inhibitors [16]. cGI-PDE has been purified from bovine heart [28] and is also well characterized for human platelets [29] and rat smooth muscle [30]. Interestingly, cyclic nucleotide-elevating agents also synergistically affect smooth muscle function [31]. However, a quantitative analysis of the biochemical events following the elevation of cyclic nucleotides in response to the combined action of synergistic platelet inhibitors has not been performed. The development of selective activators of the platelet cA-PK or cG-PK [26, 27] in combination with the recent biochemical, cell biological and molecular characterization of VASP, a novel focal adhesion protein and established substrate of both cA-PK and cG-PK in intact human platelets [18–27], enabled us to study the possible synergistic effects of platelet inhibitors on cAMP- and cGMP-mediated protein phosphorylation. Using both washed human platelets and human platelets in their physiological environment plasma, low concentrations of the nitric oxide donor SNP increased the cAMP-elevating potency of PG-$E_1$ (Fig. 4, other data not shown) confirming the results obtained with rabbit platelets [16, 17]. It is of interest to note that even small changes in cAMP levels are known to cause dramatic effects on the activity of platelet cA-PK since the intracellular cAMP concentration of unstimulated human platelets is close to the level of cA-PK cAMP-binding sites [20]. The combination of 0.1 $\mu$M SNP and 0.005 $\mu$M PG-$E_1$ caused substantial VASP serine$^{157}$ phosphorylation in both washed platelets and PRP platelets which was clearly more than additive as the extent of VASP serine$^{157}$ phosphorylation observed with these low

concentrations of SNP and PG-$E_1$ used alone (Figs 1, 2 and 4). In other experiments, preincubation of washed human platelets with the cGI-PDE inhibitor milrinone also increased the cAMP-elevating effect and VASP serine$^{157}$ phosphorylation in response to PG-$E_1$ (data not shown).

In contrast to the supra-additive effects of the combination of SNP and PG-$E_1$, incubations of washed human platelets with Sp-5,6-DCl-cBiMPS and 8-pCPT-cGMP caused VASP serine$^{157}$ phosphorylation which was approx. the same or slightly less than the sum of the extent of VASP serine$^{157}$ phosphorylation observed in response to these two cyclic nucleotide analogs used alone (Fig. 3). Previously, Sp-5,6-DCl-cBiMPS and 8-pCPT-cGMP were characterized as selective membrane-permeant activators of platelet cA-PK or cG-PK, respectively, which are not hydrolysed by PDEs and which do not affect cGMP-regulated PDEs [26, 27].

Our present data indicate that the simultaneous activation of platelet cG-PK and cA-PK results in additive effects on VASP serine$^{157}$ phosphorylation. Inhibition of cGI-PDE type III in response to cGMP-elevating nitric oxide donors further increases the extent of cAMP elevation and VASP serine$^{157}$ phosphorylation caused by low concentrations of adenylyl cyclase activators such as PG-$E_1$.

VASP phosphorylation and in particular VASP serine$^{157}$ phosphorylation closely correlates with the inhibition of both platelet and fibrinogen receptor activation [5, 13, 14, 19–24, 32, 33]. In this respect, it is of considerable interest that cyclic nucleotide-elevating agents also synergistically inhibit fibrinogen receptor activation [33]. Furthermore, platelet VASP serine$^{157}$ phosphorylation in response to physiological, endothelium-derived factors requiring both nitric oxide and prostacyclin has been observed using a reconstituted human cell culture system and an intact coronary vascular bed [23, 24]. However, VASP phosphorylation sites other than serine$^{157}$ and other cA-PK and cG-PK substrates in human platelets are certainly additional targets involved in mediating the effects of cyclic nucleotide-elevating platelet inhibitors. Another important aspect of our present work is the observation that very low levels of cGMP in human platelet are capable of inhibiting the cGI-PDE (this study, Fig. 4) despite the very high concentration of cGMP-PK known to be present in this cell type [20]. Our data therefore indicate that the cGMP-binding site of the cGI-PDE can successfully compete with the cGMP-binding site of type I cGMP-PK for the available cGMP in intact cells.

In conclusion, the synergistic inhibition of human platelets by cGMP-elevating and cAMP-elevating agents appears to consist of several components as summarized in Fig. 5:

(a) An additive component due to cG-PK- and cA-PK-mediated VASP serine$^{157}$ phosphorylation.
(b) A supra-additive component due to the cGMP-caused, cGI-PDE-mediated potentiation of the cAMP response and VASP serine$^{157}$ phosphorylation by the cA-PK.
(c) Phosphorylation by cG-PK and/or cA-PK of substrates and sites other than VASP serine$^{157}$

The possible regulatory role of cA-PK-mediated phosphorylation and associated activation of the cGI-PDE in intact cells needs further investigation.

Further elucidation of the interaction of vasoactive drugs at the molecular level using human platelets and other human vascular and cardiac cell types should be helpful for the development of better and safer antithrombotics, antihypertensive agents and other cardiovascular drugs.

*Acknowledgements*—This study was supported by the Deutsche Forschungsgemeinschaft (Ko 210/11-3 and SFB 355). The authors thank Dr Imme Haubitz for advice and help with the statistics and S. Ebert for art work and help in the preparation of this manuscript.

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