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# COMPARISON OF VASODILATORY PROSTAGLANDINS WITH RESPECT TO cAMP-MEDIATED PHOSPHORYLATION OF A TARGET SUBSTRATE IN INTACT HUMAN PLATELETS

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**Abstract**—The recent purification of a vasodilator-stimulated phosphoprotein (VASP) from human platelets and the development of a specific antiserum against VASP made it possible to study the quantitative effects of cAMP-elevating prostaglandins on cAMP-mediated phosphorylation of VASP in intact human platelets. Prostacyclin (PG-$I_2$), prostaglandin-$E_1$ (PG-$E_1$) and the stable prostacyclin-analog Iloprost, all agents used for the treatment of peripheral vascular disease, induced rapid, stoichiometric and reversible phosphorylation of VASP in human platelets mediated by the cAMP-dependent protein kinase. However, there were substantial differences between these three cAMP-elevating prostaglandins with respect to their effects on extent, duration and reversibility of VASP phosphorylation. Maximal VASP phosphorylation was induced both by PG-$I_2$ and Iloprost, but the PG-$I_2$ effect was only of short duration in comparison to that of Iloprost. The extent of PG-$E_1$-induced VASP phosphorylation was less than that observed with PG-$I_2$ and Iloprost. In endothelial cell-platelet coincubations, an endothelial cell-derived, indomethacin-sensitive factor caused a rapid elevation of platelet cAMP level and VASP phosphorylation. These results provided direct evidence that human endothelial cells are capable of producing biologically active quantities of cAMP-elevating prostaglandins sufficient to induce stoichiometric cAMP-mediated protein phosphorylation in human platelets. VASP-phosphorylation induced by PG-$I_2$ and PG-$E_1$ was completely reversible after removal of the prostaglandins whereas this was only partially the case with Iloprost. In addition, evidence is presented that the prostaglandin-regulated adenylate cyclase system but not the cAMP-mediated protein phosphorylation desensitizes in human platelets after prolonged treatment with cAMP-elevating prostaglandins. VASP phosphorylation is proposed as a marker for quantitating aspects of vessel wall–platelet interaction.

Following the discovery of prostacyclin (PG-$I_2$) as a potent endothelial cell-derived vasodilator and platelet inhibitor [1], PG-$I_2$ and related prostaglandins were intensely studied with respect to clinical implications, pharmacology and potential use as therapeutic agents [2, 3]. Because of their potent inhibitory effects on both vascular smooth muscle contraction and platelet aggregation, intra-arterial and intravenous infusions with PG-$I_2$, prostaglandin-$E_1$ (PG-$E_1$) and the stable PG-$I_2$-analog Iloprost have been evaluated as therapy for severe arterial occlusive disease, Raynaud's syndrome and certain other diseases [2, 4–6]. PG-$I_2$, PG-$E_1$ and Iloprost have also been used for ischemic heart disease, including acute myocardial infarction, with variable clinical results [7–11]. Despite the chemical instability and very short biological half-life of these agents in man, short-term PG-$E_1$- or PG-$I_2$-infusions have produced dramatic and surprisingly long-lasting functional and symptomatic improvements in severe peripheral vascular disease [2, 4–6]. Apparently, these vasodilatory prostaglandins are capable of inducing long-lasting biochemical events in the vasculature. However, very little is known about the mechanism of action of these prostaglandins at the cellular or molecular level. The intracellular effects of PG-$I_2$, PG-$E_1$ and Iloprost appear to be mediated by cAMP and cAMP-dependent protein kinase [2, 12] however, with the exception of cAMP level determinations, no quantitative biochemical data exist which clarify the intracellular effects of cAMP-elevating vasodilators. Recently, a 50 kDa protein of human platelets has been characterized which is phosphorylated in intact platelets in response to cAMP- and cGMP-elevating vasodilators and in platelet membranes by endogenous cAMP- and cGMP-dependent protein kinase [13]. Evidence suggests that this vasodilator-stimulated phosphoprotein (VASP†) plays an important role in cyclic nucleotide-mediated inhibition of platelet aggregation [12]. Therefore VASP has been purified [14] and an immunological method was subsequently developed for studying the quantitative aspects of VASP phosphorylation using intact human platelets [15]. Here we report the quantitative analysis of the effects of PG-$I_2$, PG-$E_1$ and Iloprost on cAMP levels and on VASP phosphorylation, dephosphorylation and rephosphorylation using intact human platelets, one of the principal targets of these vasodilatory prostaglandins.

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† Abbreviation: VASP, vasodilator-stimulated phosphoprotein.

## MATERIALS AND METHODS

*Materials.* [$^{125}$I]Protein A (30 mCi/mg) and the

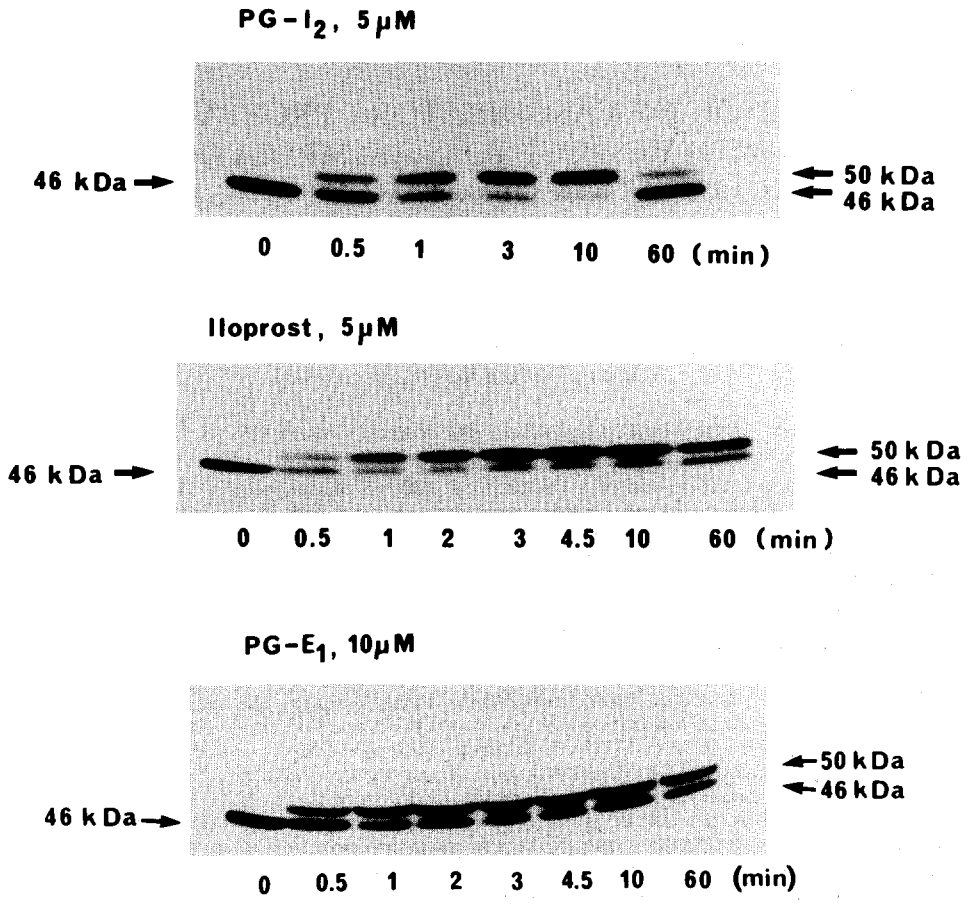



Fig. 1. Autoradiograph showing the time course of VASP phosphorylation with intact human platelets in response to 5 $\mu$M PG-$I_2$ (upper panel), 5 $\mu$M Iloprost (middle panel) and 10 $\mu$M PG-$E_1$ (lower panel). Washed human platelets ($1 \times 10^9$ cells/mL) were incubated with PG-$I_2$, Iloprost or PG-$E_1$. Aliquots ($0.6 \times 10^8$ platelets) were removed at the times indicated, immediately mixed with an SDS-containing stop solution and boiled. VASP phosphorylation was analysed by the Western blot technique described in Materials and Methods. The positions of dephospho-VASP (46 kDa) and phospho-VASP (50 kDa) are indicated.

[$^{125}$I]cAMP assay system were obtained from Amersham Buchler (Braunschweig, F.R.G.). Prostacyclin (PG-$I_2$; trade name Flolan) was obtained from Wellcome (Beckenham, U.K.).

Storage and preparation of stock solutions were done using the manufacturer's recommendations and buffers. The diluent buffer for PG-$I_2$ consisted of 0.147% (w/v) NaCl and 0.188% (w/v) glycine (pH 10.5). PG-$I_2$ was used in experiments immediately after the appropriate dilutions were made. The prostacyclin-analog Iloprost was a gift provided by Schering (Berlin, F.R.G.) in vials containing 0.1 mg/mL Iloprost (277 $\mu$M) in Tris-buffered saline (pH 8.4). The vials were stored at room temperature. Further dilutions were prepared using the incubation buffer on the day of the experiment and subsequently stored at −20°.

Prostaglandin $E_1$ (PG-$E_1$) was purchased from Sigma (Deisenhoten, F.R.G.). A stock solution of 10 mM PG-$E_1$ was prepared using ethanol, stored at −20° and diluted further using the incubation buffer on the day of the experiment.

Preparation and characterization of the antiserum against VASP has been published [14, 15]. Other chemicals and materials were from commercial sources as described previously [15].

*Isolation of platelets.* Washed human platelets were prepared essentially as described [13] with minor modifications. Platelet rich plasma was centrifuged for 10 min at 700 *g*, and the platelets were resuspended in an isotonic isolation buffer containing 10 mM Hepes (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 5.5 mM glucose and 1 mM EDTA. Platelets were washed twice using the same buffer and centrifugation conditions (10 min, 700 *g*). These washed platelets were finally resuspended in the same isotonic isolation buffer to yield a density of $1 \times 10^9$ cells/mL ($1.5 \times 10^9$ cells/mL for endothelial cell–platelet coincubation experiments).

*Analysis of cAMP levels, protein phosphorylation and aggregation of washed human platelets.* Washed platelets were incubated with the various prostaglandins at 37°, and aliquots were removed from the suspension at the time points indicated (see figures).

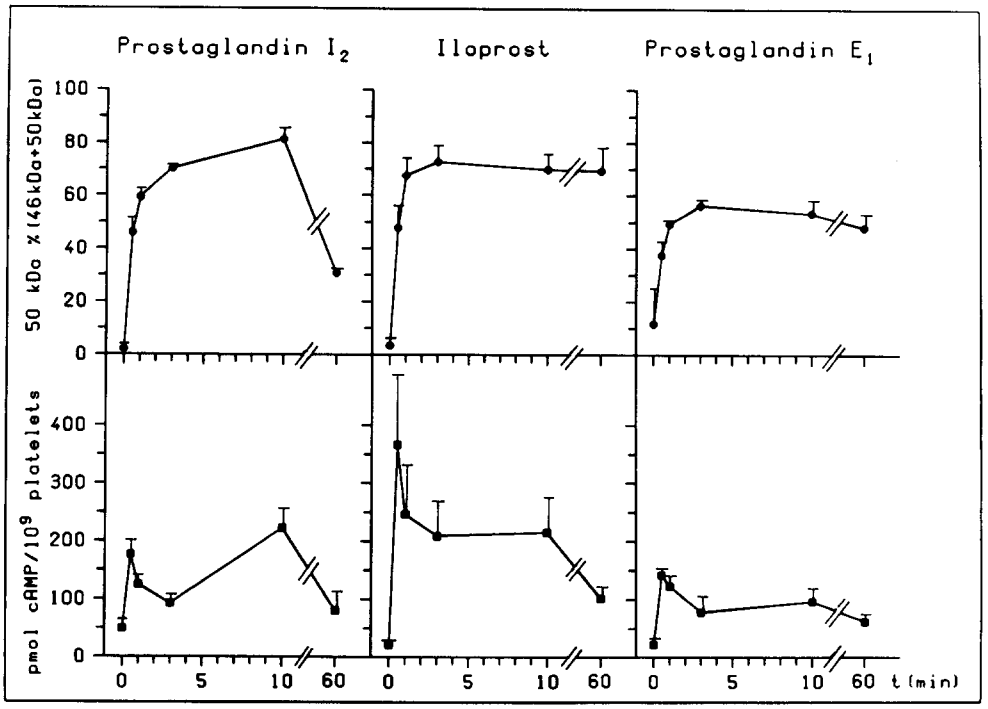



Fig. 2. Time course of the effects of high concentrations of various prostaglandins on VASP phosphorylation (upper panel) and cAMP levels (lower panel) in intact human platelets. Washed platelets ($1 \times 10^9$ cells/mL) were incubated with 5 $\mu$M PG-$I_2$, 5 $\mu$M Iloprost or 10 $\mu$M PG-$E_1$. Two sets of aliquots were withdrawn at the times indicated. One set of aliquots ($0.6 \times 10^8$ platelets) was mixed with an SDS-containing stop solution, boiled and analysed for VASP phosphorylation by Western blots. Other aliquots ($2 \times 10^8$ platelets) were mixed with an equal volume of 20% trichloroacetic acid and analysed for their cAMP-content by radioimmunoassay as described in Materials and Methods. Phosphorylation of VASP is expressed as appearance of phospho-VASP (50 kDa protein) as per cent of total VASP (46 kDa + 50 kDa protein). Data shown represent the means (±SD) of three separate experiments.

For the analysis of cAMP, aliquots containing $2 \times 10^8$ platelets were immediately mixed with an equal volume of ice-cold 20% trichloroacetic acid. Extraction of cAMP and subsequent measurements by radioimmunoassay was carried out as described previously [15]. For the analysis of protein phosphorylation, aliquots containing $0.6 \times 10^8$ platelets were mixed with an SDS-containing stop solution, boiled and subsequently analysed for the extent of VASP phosphorylation, as described previously [15]. Briefly, proteins of the aliquots were separated by 9% SDS–polyacrylamide gels, blotted to nitrocellulose sheets (Western blotting) and radiolabelled using an antiserum against VASP and [$^{125}$I]protein A. Dephospho-VASP (46 kDa) and phospho-VASP (50 kDa) were localized by autoradiography and quantitated by cutting out and counting the radioactive [$^{125}$I]protein A-labelled bands. Dephospho-VASP (46 kDa) and phospho-VASP (50 kDa) are each expressed as percentage of total VASP (46 + 50 kDa).

When the reversibility of prostaglandin effects was studied, platelets were incubated with the prostaglandins for the times indicated (see figures) and prostaglandins were removed from the medium by washing the platelets twice with the isotonic incubation buffer as described above for the isolation of cells. Prostaglandins were added to the platelet suspension at the times indicated (see figures).

For platelet aggregation studies, cells were isolated as described above except that the platelets were finally resuspended in an isotonic phosphate incubation buffer (pH 7.4) containing 4.3 mM $K_2HPO_4$, 4.3 mM $Na_2HPO_4$, 24.4 mM $NaH_2PO_4$, 113 mM NaCl and 5.5 mM glucose. Furthermore, 1 mM $CaCl_2$ plus 2 mM $MgCl_2$ was added to the final suspension. Platelets were incubated in the presence or absence of prostaglandins for 2–5 min at 37° before the aggregation was induced by the addition of 1 unit/mL thrombin. Preincubation and aggregation was monitored using the platelet aggregation profiler PAP 4 from Bio Data Corporation.

*Endothelial cell–platelet coincubations.* Human umbilical vein endothelial cells were isolated and cultured as described previously [16]. The purity of endothelial cells (more than 95%) was verified by light microscopy (typical "cobblestone" morphology) and by fluorescence microscopy (expression of von Willebrand factor and uptake of acetylated low-density lipoprotein) as described previously [17, 18]. Endothelial cells of passages 1–3 were used for

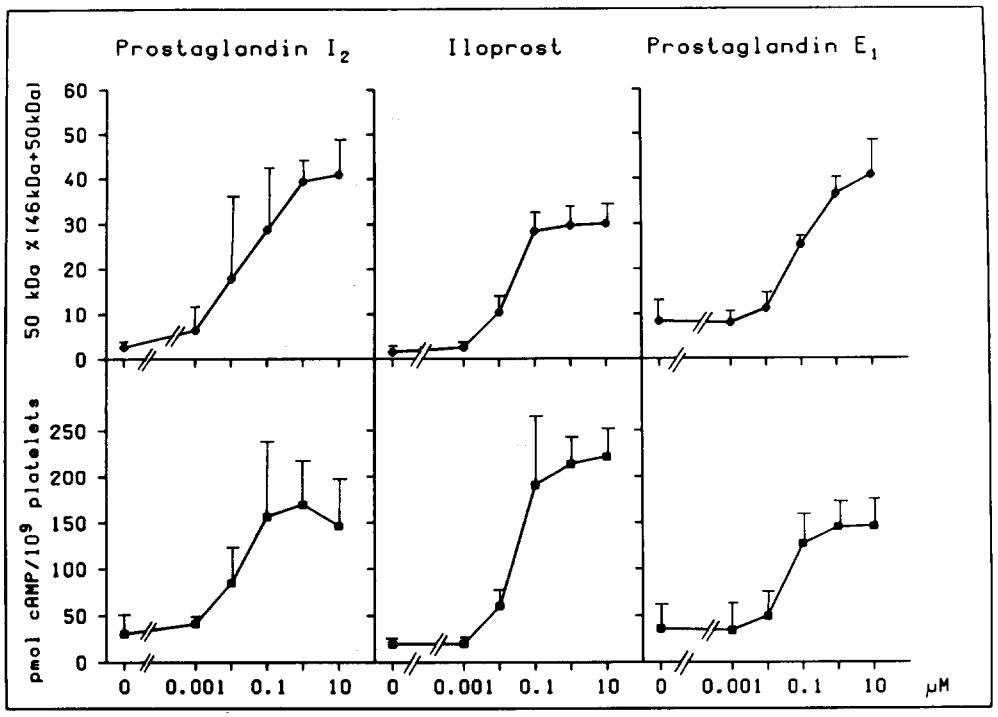



Fig. 3. Concentration-dependent effects of PG-$I_2$, Iloprost and PG-$E_1$ on VASP phosphorylation (upper panel) and cAMP levels (lower panel) in intact platelets. Washed platelets were incubated with the indicated concentrations of PG-$I_2$, Iloprost or PG-$E_1$ for 20 sec. Aliquots were analysed for VASP phosphorylation or cAMP content as described in the legend of Fig. 2. The results represent the means (±SD) of three separate experiments.

experiments. Washed human platelets (final density $1.5 \times 10^9$ cells/mL) and monolayers of endothelial cells were prepared as described previously [13, 16]. Coincubations were initiated by the addition of $0.9 \times 10^9$ platelets (600 μL) to the endothelial cells to yield a final platelet density of $0.75 \times 10^9$ cells/mL. In certain experiments, platelets and endothelial cells were preincubated separately with 10 μM indomethacin for 1 hr at 37° before incubations were started. Aliquots of platelets ($4.5 \times 10^7$ platelets) were removed before (0 min) and after (3 min) the coincubations for the analysis of platelet cAMP levels and VASP phosphorylation carried out as described above. At this time, analysis of the protein concentration of the platelet suspension indicated that no significant loss of platelets due to adhesion occurred during these coincubations.

## RESULTS

The effects of high concentrations of PG-$I_2$ (5 μM), Iloprost (5 μM) and PG-$E_1$ (10 μM) on VASP phosphorylation and cAMP levels in platelets are demonstrated in the Western blot of Fig. 1 and by further quantitative analysis in Fig. 2. Addition of all three prostaglandins to washed platelets caused the rapid conversion of about 75% (with PG-$I_2$ and Iloprost) or 55% (with PG-$E_1$) of VASP to the 50 kDa phospho-form within 3 min (Figs 1 and 2). Within 60 min of the addition of these prostaglandins to platelets, most of the VASP was converted back to the 46 kDa dephospho-form during the incubations with PG-$I_2$ but not during the incubations with Iloprost and PG-$E_1$. Phosphorylation of VASP was preceded by a rapid 5–10-fold increase of the platelet cAMP content. The cellular level of cAMP reached a peak within 30 sec of the addition of the prostaglandins then declined but remained somewhat elevated even at the end of the 60 min incubation (Fig. 2). Consistently, cAMP levels rose again at a second time point observed 10 min after the addition of 5 μM PG-$I_2$, but not Iloprost or PG-$E_1$ (Fig. 2).

Using 20-sec incubations with the three prostaglandins, half-maximal effects on platelet cAMP level and VASP phosphorylation were observed with 20–30 nM PG-$I_2$ or Iloprost and with 50–100 nM PG-$E_1$ (Fig. 3). These data were also compared with the effects of PG-$I_2$, Iloprost and PG-$E_1$ on the aggregation of washed platelets. Half-maximal inhibitory effects on thrombin-induced aggregation of washed platelets were observed with 20–50 nM Iloprost, 50–100 nM PG-$E_1$ and 200–500 nM PG-$I_2$ (data not shown). The time course of the effects of low concentrations of PG-$I_2$, Iloprost and PG-$E_1$ (10 nM in all cases) on platelet cAMP level and VASP phosphorylation was also analysed (Fig. 4). Under these experimental conditions, both PG-$I_2$ and Iloprost caused a more than 5-fold increase in the cAMP-level and a more than 80% conversion of VASP from the dephospho-form to the phospho-form, whereas PG-$E_1$ had only moderate effects on

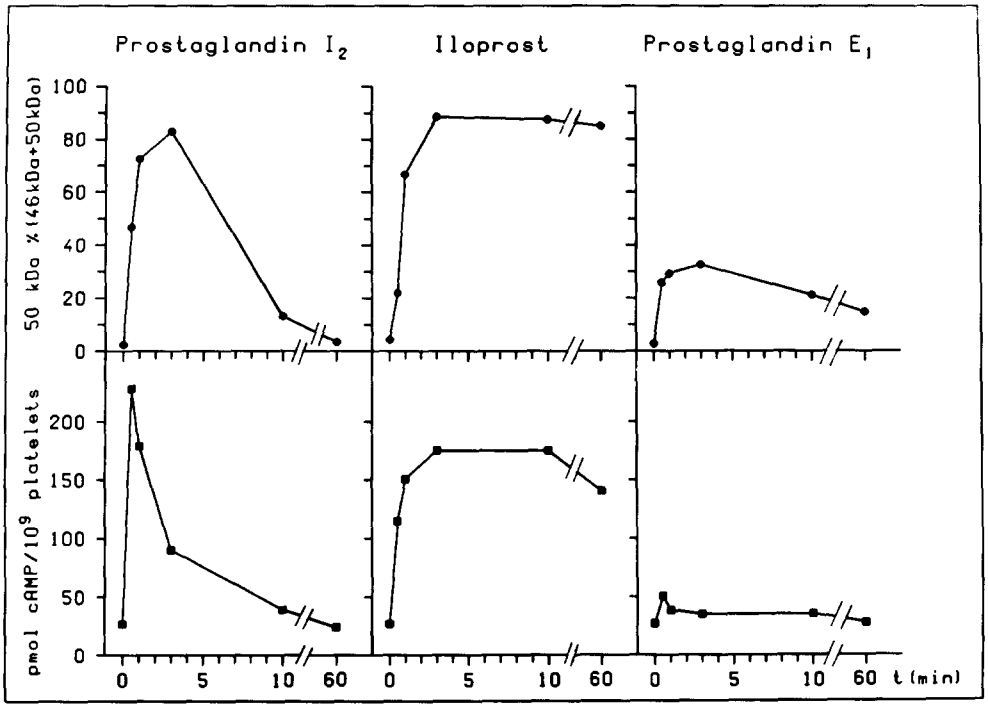



Fig. 4. Time course of the effects of low concentrations of various prostaglandins on VASP phosphorylation (upper panel) and cAMP levels (lower panel) in intact human platelets. Washed platelets were incubated with 0.01 $\mu$M PG-$I_2$, 0.01 $\mu$M Iloprost or 0.01 $\mu$M PG-$E_1$. Aliquots were withdrawn at the times indicated for the analysis of VASP phosphorylation or cAMP content as described in the legend of Fig. 2. The data shown are from one experiment. Similar data were obtained in a second experiment.

the cAMP level (about 2-fold elevation) and VASP phosphorylation (about 30%). The effects induced by 10 nM Iloprost were stable for at least 60 min whereas the PG-$I_2$-induced effects on platelet cAMP content and VASP phosphorylation rapidly returned to near basal level within 10 min after the initial addition of PG-$I_2$ (Fig. 4). In contrast to the results with high concentrations of PG-$I_2$ (Fig. 2), the second cAMP rise (10 min after the addition of PG-$I_2$) was not observed with low concentrations (10 nM) of PG-$I_2$ (Fig. 4). The moderate effects of 10 nM PG-$E_1$ partially declined within 60 min (Fig. 4).

The question of whether human endothelial cells are capable of producing biologically active prostaglandin concentrations sufficient to cause cAMP-mediated VASP phosphorylation in intact human platelets was investigated using coincubations of human umbilical vein endothelial cells and washed platelets. Within 3 min of such coincubations, endothelial cell-derived factors caused an 8-fold elevation of platelet cAMP level and a 15-fold increase in phospho-VASP in intact platelets (Table 1). Pretreatment of endothelial cells with indomethacin abolished the elevation of platelet cAMP level and inhibited about two-thirds of the endothelial cell-induced platelet VASP phosphorylation (Table 1).

The potential reversibility of the effects of high concentrations of prostaglandins on platelet cAMP content and VASP phosphorylation was also investigated. The prostaglandins and their possible metabolites were removed from the incubation medium by washing the platelets twice (centrifugation/resuspension) with an incubation buffer without prostaglandins. With both PG-$I_2$ and PG-$E_1$, a rapid return of the platelet cAMP content to basal levels followed by a complete dephosphorylation of VASP was observed when the prostaglandins had been removed from the medium (Fig. 5). In contrast, cAMP levels and VASP phosphorylation remained highly elevated for at least 60 min after the removal of Iloprost from the medium (Fig. 5). When a similar experiment was carried out with 10 nM Iloprost, elevated cAMP levels returned to near basal levels and 50% of phospho-VASP to dephospho-VASP within 60 min after the removal of Iloprost (data not shown).

Following an incubation of platelets with prostacyclin and subsequent removal of this prostaglandin and its metabolites from the medium, newly added PG-$I_2$ could restimulate the elevation of cAMP and VASP phosphorylation in these platelets (Fig. 6). Although the second stimulation with PG-$I_2$ again caused maximal VASP phosphorylation, it elevated the cAMP content to only 50% of the level observed after the first stimulation (Fig. 6). When similar experiments with platelets (prostaglandin incubation, removal and second incubation) were carried out using low concentrations of PG-$I_2$, Iloprost and PG-$E_1$ (10 nM in all cases), VASP phosphorylation and elevation of cAMP levels were observed after the second addition of all three prostaglandins (data not

Table 1. Endothelial cell-dependent regulation of cAMP levels and VASP phosphorylation in human platelets

| Conditions | Phospho-VASP (%) (50 kDa/46 kDa + 50 kDa) | cAMP (pmol/$10^9$ platelets) |
|---|---|---|
| −EC/0 min | 4.5 ± 2.1 | 22.2 ± 6.8 |
| −EC/3 min | 6.1 ± 1.8 | 26.7 ± 6.8 |
| +EC/3 min | 75.2 ± 8.7 | 167.8 ± 80.6 |
| +EC + Indo/3 min | 28.9 ± 11.1 | 25.5 ± 5.0 |

Washed human platelets were incubated with (+EC) or without (−EC) endothelial cells for 3 min. When indicated (+Indo), endothelial cells and platelets had been preincubated with 10 μM indomethacin for 1 hr.

Platelet aliquots were removed at the beginning (0 min) and end (3 min) of the incubation for the determination of platelet cAMP levels and VASP phosphorylation as described in the legend of Fig. 2.

Data represent the means (±SD) of three separate experiments.

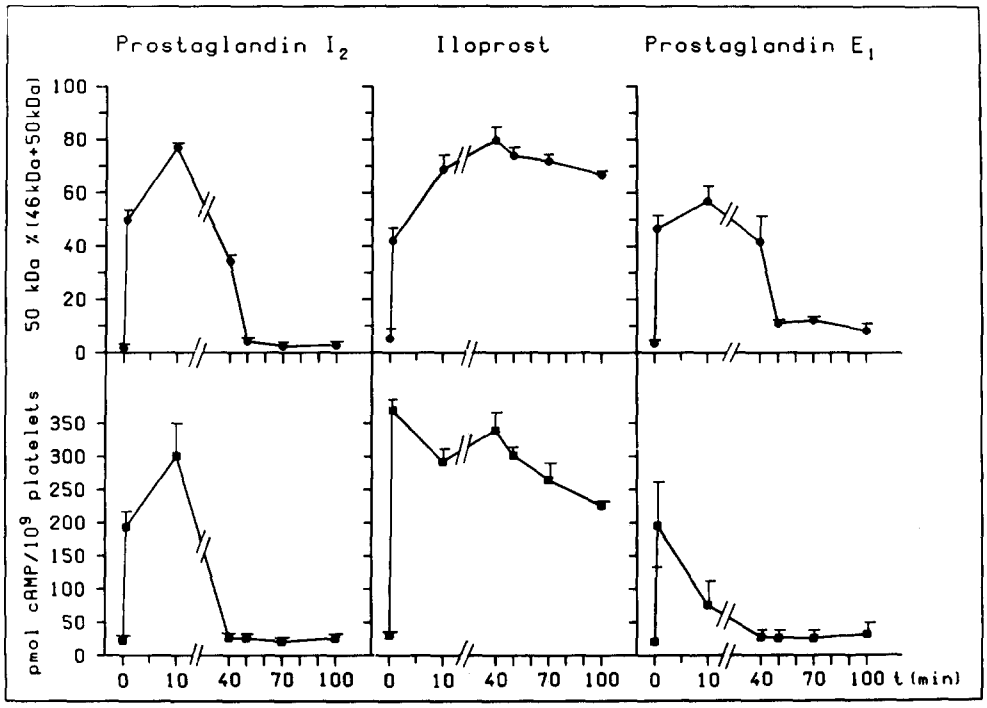
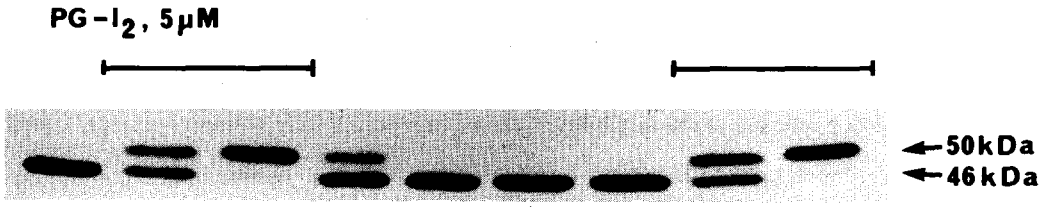



Fig. 5. Reversibility of the effects of PG-$I_2$, Iloprost or PG-$E_1$ on VASP phosphorylation (upper panel) and cAMP levels (lower panel) in intact human platelets. Washed human platelets were incubated with 5 μM PG-$I_2$, 5 μM Iloprost or 10 μM PG-$E_1$ for 10 min. Prostaglandins in the medium were then removed by washing the platelets twice with buffer for about 30 min. The incubation was then continued. Aliquots were removed for the analysis of VASP phosphorylation and cAMP content as described in the legend of Fig. 2. Data shown represent the means (±SD) of three separate experiments.

shown). However, the extent of cAMP elevation after the second incubation was only about 50% of the extent observed after the first incubation with all three prostaglandins (data not shown).

## DISCUSSION

Pharmacologically high concentrations of PG-$I_2$, Iloprost and PG-$E_1$ were used to evaluate the possible maximal effects on cAMP levels and cAMP-dependent protein phosphorylation in intact human platelets. All three prostaglandins rapidly elevated the intracellular level of cAMP and caused the phosphorylation of VASP (Figs 1 and 2). Whereas PG-$I_2$ and Iloprost almost completely quantitatively converted VASP from the 46 kDa dephospho-form to the 50 kDa phospho-form, PG-$E_1$ (even under maximal conditions) was unable to convert more than about 55% of VASP to the phospho-form (Figs




| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0.5 | 10 | 40 | 50 | 70 | 100 | 0.5 | 10 | min |
| 2 | 50 | 77 | 34 | 4 | 2 | 3 | 53 | 77 | % 50k Da |
| 22 | 194 | 301 | 27 | 26 | 21 | 26 | 148 | 143 | pmol cAMP/ $10^9$ platelets |

Fig. 6. Autoradiograph and quantitative analysis demonstrating that PG-$I_2$ effects on VASP phosphorylation and cAMP levels in intact human platelets can be reversed and restimulated. Washed human platelets were incubated for 10 min with 5 μM PG-$I_2$. Prostaglandins were subsequently removed from the medium by washing the platelets as described in the legend to Fig. 5, which required the time between 10 and 40 min of the entire incubation period. PG-$I_2$ (5 μM) was added again, 100 min after the start of the incubation, to the platelet suspension which was then incubated for an additional 10 min. Aliquots were removed for the analysis of VASP phosphorylation and cAMP content as described in the legend to Fig. 2. The upper panel demonstrates the phosphorylation of VASP in one experiment with the incubation time indicated below the autoradiograph. The quantitative analysis of VASP phosphorylation (per cent 50 kDa protein of total VASP) and cAMP content in platelets removed at the times indicated is shown in the lower panel. The data represent the means of three separate experiments. The standard deviations (not shown here for lack of space) were similar to those in the experiments of Figs 2, 3 and 4.

1, 2, 4 and 5). In the constant presence of high and low concentrations of prostaglandins, the effect of Iloprost on VASP-phosphorylation was stable for at least 60 min, while the effects of PG-$E_1$ were partially and those of PG-$I_2$ almost completely reversed (Figs 2 and 4). Similar effects were found when platelet cAMP levels were studied.

In the various experiments, the basal cAMP concentration of washed, untreated human platelets was about 20–40 pmol/$10^9$ cells which corresponds to an intracellular concentration of about 4–8 μM assuming a mean platelet volume of about 5 fL [19] and an uncompartmentalized soluble distribution of the cyclic nucleotide. Interestingly, the basal cAMP concentration corresponds reasonably well with the concentration of cAMP-binding sites due to the cAMP-dependent protein kinase which was recently estimated to be 6–7 μM in human platelets (Ref. 15; Eigenthaler *et al.*, manuscript in preparation). This similarity in the concentration of platelet cAMP and cAMP-binding sites would predict that relatively small changes in the cAMP level would be sufficient to activate the cAMP-dependent protein kinase leading to VASP phosphorylation. Indeed, significant VASP phosphorylation was already observed with only 2-fold increases in the platelet cAMP level (Fig. 2–4). High concentrations of PG-$I_2$, Iloprost and PG-$E_1$ maximally raised the platelet cAMP level about 8-, 18- and 7-fold, respectively. Half-maximal effects on cAMP levels and VASP phosphorylation (using an incubation time of 20 sec) were observed with 20–30 nM PG-$I_2$ and Iloprost and with 50–100 nM PG-$E_1$ (Fig. 3). These data agree reasonably well with our observation (data not shown) that thrombin-induced platelet aggregation was half-maximally inhibited with 20–50 nM Iloprost, 50–100 nM PG-$E_1$ and 200–500 nM PG-$I_2$. Half-maximal inhibition of platelet aggregation was observed with even lower PG-$I_2$ concentration when the preincubation time in these measurements was reduced to 30 sec. This suggests that the apparent discrepancy between the half-maximal effects of PG-$I_2$ on cAMP level/VASP phosphorylation and its effects on platelet aggregation is most likely due to the chemical instability of PG-$I_2$ [1, 2] and the rapid reversibility of the biological effects of PG-$I_2$ in platelets (Fig. 4) together with the longer preincubation time for the analysis of platelet aggregation. A similar rank order of potency with respect to the effects of PG-$I_2$, Iloprost and PG-$E_1$ on platelet aggregation and cAMP level has been observed previously [20]. In so far as receptor binding studies have been performed and carefully analysed, the half-maximal effects of PG-$I_2$ and Iloprost on platelet cAMP level and VASP phosphorylation agree very well with the binding characteristics of the putative PG-$I_2$/Iloprost receptor which apparently has a single class of binding sites with a $K_d$ of 10–20 and 20–30 nM for Iloprost and PG-$I_2$, respectively [21, 22]. This information and the results of our present study suggest that the binding of cAMP-elevating prostaglandins to their receptors and the subsequent activation of adenylate cyclase and cAMP-dependent protein kinase are tightly coupled in human platelets.

There were significant differences between the

three prostaglandins studied with respect to their effects on platelet cAMP level and VASP phosphorylation. The rapid reversibility of the effects of PG-$I_2$ despite the constant presence of this agent (Figs 1, 2 and 4) is most likely due to the instability of PG-$I_2$.

The long-term effects of 5 $\mu$M and 10 nM Iloprost on cAMP levels and VASP phosphorylation demonstrates the well established chemical and biological stability of this prostacyclin analog. These results also demonstrate that cAMP-mediated protein phosphorylation in human platelets remains elevated as long as cAMP levels are high enough to keep the cAMP-dependent protein kinase activated. This interpretation is also supported by the results of the experiments in which the prostaglandins were removed from the platelet suspension (wash-out experiments). With both PG-$E_1$ and PG-$I_2$, removal of the prostaglandins from the medium resulted in a rapid return of the cAMP concentration to basal levels followed by the complete dephosphorylation of VASP (Figs 5 and 6). These results suggest that the return of the cAMP concentration to basal levels is rapidly followed by the inactivation of the cAMP-dependent protein kinase and complete dephosphorylation of VASP by the appropriate phosphatase.

The relatively slow return of elevated cAMP levels and VASP phosphorylation to basal conditions after removal of Iloprost from the medium is consistent with the chemical and biological stability of this analog and with the low dissociation rate constant of the prostacyclin/Iloprost receptor [22].

After a first incubation of human platelets with PG-$E_1$, Iloprost or PG-$I_2$ and subsequent removal of these agents from the medium, cAMP levels and VASP phosphorylation could again be elevated by a second addition to these prostaglandins (Fig. 6; other data not shown). However, whereas the full extent of VASP phosphorylation could be obtained in response to the second addition, cAMP levels under these experimental conditions could only be elevated to about 50% of the level of the first incubation (Fig. 6; other data not shown). This is consistent with observed desensitization of the platelet prostacyclin/adenylate cyclase system after exposure to cAMP-elevating prostaglandins [22]. In contrast, desensitization of the intracellular signal transduction system activated by cAMP apparently does not occur, and the full extent of VASP phosphorylation is obtained even with relatively small increases in the cAMP level.

In many different experiments, the extent of VASP phosphorylation using saturating concentrations of prostaglandin was consistently higher with PG-$I_2$ and Iloprost when compared with PG-$E_1$ (Figs 1, 2 and 5). This could be due to a possible cellular heterogeneity of human platelets [19], receptor heterogeneity for PG-$E_1$/PG-$I_2$ [21–27] or a differing compartmentalization of the PG-$E_1$- and PG-$E_2$-induced protein phosphorylation.

It is also necessary to compare our *in-vitro* data on human platelets with the physiological and pharmacological concentrations of cAMP-elevating prostaglandins found in humans. Because of the chemical lability of PG-$I_2$ it has been very difficult to estimate the circulating concentration of this prostaglandin [2, 3]. However, it was reported recently [28] that biologically effective PG-$I_2$ concentrations as high as 305 fg/$\mu$L (about 0.8 nM) can be produced locally at sites of vascular damage. Pharmacokinetic studies using intravenous infusions with hemodynamically effective dosages of 5–15 ng PG-$E_1$/kg/min [29] or 1–3 ng Iloprost/kg/min [30] revealed that circulating levels of 200–400 fg/$\mu$L PG-$E_1$ (about 0.5–1.0 nM) and 46–135 fg/$\mu$L Iloprost (about 0.13–0.40 nM) may be obtained under those conditions [29, 30]. Therefore, it seems reasonable to assume that a concentration of 0.1–1 nM cAMP-elevating prostaglandins is required in man for the hemodynamic, pharmacological and clinical effects observed which may be achieved with various dosage regimens [31].

Interestingly, demonstration of platelet cAMP level elevation and *ex-vivo* inhibition of platelet aggregation following these various prostaglandin infusions has been possible with Iloprost [30, 32] but is difficult with PG-$I_2$ or PG-$E_1$, despite the fact that small effects on platelet cAMP level and evidence for *in-vivo* platelet inhibition has been obtained for the latter two prostaglandins as well [2, 4, 33]. This would then indicate that the *in-vivo* inhibition of platelet activation obtained with concentrations of cAMP-elevating prostaglandins in the range of 0.1–1 nM can be detected in *ex-vivo* platelet function tests only in the case of Iloprost due to the long-lasting effect of this agent. This interpretation is consistent with extent, duration and reversibility of the prostaglandin-induced VASP phosphorylation reported in our present study. However, lower concentrations of prostaglandins are apparently required to observe *in-vivo* platelet inhibition (0.1–1 nM) than to observe biochemical effects with platelets *in vitro*, since the threshold concentrations required to detect effects of PG-$I_2$, Iloprost or PG-$E_1$ on cAMP level and VASP phosphorylation with washed platelets were in the order of 1–10 nM (Fig. 4). Additional factors may increase or potentiate the effects of cAMP-elevating prostaglandins on human platelets *in vivo*. This is very likely because of the observation that cAMP-elevating vasodilators (i.e. PG-$I_2$, PG-$E_1$, Iloprost etc.) and cGMP-elevating vasodilators (i.e. nitroprusside, endothelium-derived relaxing factor etc.) have *in-vitro* synergistic effects with respect to inhibition of platelet aggregation [34–36] and platelet VASP phosphorylation (Ref. 15; Nolte *et al.*, unpublished experiments).

Coincubation experiments using human endothelial cells and washed human platelets demonstrated that endothelial cells are capable of producing quantities of cAMP-elevating prostaglandins sufficient to cause the cAMP-mediated phosphorylation of VASP in intact platelets. Endothelial cell-derived factors caused a rapid, 8-fold elevation of platelet cAMP level and converted most of the VASP to the phospho-form, effects strongly blocked by the cyclooxygenase inhibitor indomethacin (Table 1). These data indicated that these effects were mediated by a cAMP-elevating, endothelial cell-derived prostaglandin, most likely PG-$I_2$. Interestingly, indomethacin abolished the endothelial cell-induced platelet cAMP response, but did not completely

inhibit platelet VASP phosphorylation (Table 1). A whole series of additional experiments (Nolte *et al.*, manuscript in preparation) provided evidence that human endothelial cells cause platelet VASP phosphorylation, not only by PG-$I_2$/cAMP but also by a mechanism involving endothelium-derived relaxing factor and its signal transduction by cGMP/cGMP-dependent protein kinase.

In conclusion, significant differences between PG-$I_2$, PG-$E_1$ and Iloprost were observed with respect to their effects on extent, duration and reversibility of VASP phosphorylation in intact human platelets. The observation that PG-$I_2$ causes stoichiometric but rapidly reversible VASP phosphorylation can be considered to be a biochemical correlate for the practical experience [2] that PG-$I_2$ is very useful for the preparation of functional platelets as well as for the complete short-term inhibition of platelet activation. In contrast, Iloprost may be more useful for long-term platelet inhibition. PG-$E_1$ may activate a different receptor population than PG-$I_2$ and Iloprost, and the duration of PG-$E_1$-induced effects on platelets is longer than those of PG-$I_2$, but shorter than those of Iloprost. However, our data do not readily explain why long-lasting improvements of vascular disease is observed after short-term prostaglandin infusions. This may involve long-term effects resulting from inhibition of platelet-derived growth factor release by cAMP-elevating prostaglandins, and/or long-term effects of these prostaglandins on other vascular cells including smooth muscle cells. Nevertheless, VASP phosphorylation in intact platelets appears to be a useful indicator for the quantitative effects of cAMP- and cGMP-elevating vasodilators. In this respect, analysis of VASP phosphorylation is more sensitive than the analysis of platelet cAMP level (i.e. a transient 2-fold elevation of cAMP resulted in a long-lasting, several-fold elevation of phospho-VASP, Fig. 4). VASP phosphorylation could potentially become a clinical marker for vessel wall–platelet interactions, especially those mediated by PG-$I_2$ and endothelium-derived relaxing factor. Such markers are certainly needed [37, 38] and our efforts to analyse the state of VASP phosphorylation in platelets, rapidly isolated from the whole blood of patients, are in progress.

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