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# Time-dependent inhibitory effects of cGMP-analogues on thrombin-induced platelet-derived microparticles formation, platelet aggregation, and P-selectin expression

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ABSTRACT

In platelets, nitric oxide (NO) activates cGMP/PKG signalling, whereas prostaglandins and adenosine signal through cAMP/PKA. Cyclic nucleotide signalling has been considered to play an inhibitory role in platelets. However, an early stimulatory effect of NO and cGMP-PKG signalling in low dose agonist-induced platelet activation have recently been suggested. Here, we investigated whether different experimental conditions could explain some of the discrepancy reported for platelet cGMP-PKG-signalling. We treated gel-filtered human platelets with cGMP and cAMP analogues, and used flow cytometric assays to detect low dose thrombin-induced formation of small platelet aggregates, single platelet disappearance (SPD), platelet-derived microparticles (PMP) and thrombin receptor agonist peptide (TRAP)-induced P-selectin expression. All four agonist-induced platelet activation phases were blocked when platelets were costimulated with the PKG activators 8-Br-PET-cGMP or 8-pCPT-cGMP and low-doses of thrombin or TRAP. However, extended incubation with 8-Br-PET-cGMP decreased its inhibition of TRAP-induced P-selectin expression in a time-dependent manner. This effect did not involve desensitisation of PKG or PKA activity, measured as site-specific VASP phosphorylation. Moreover, PKG activators in combination with the PKA activator *Sp*-5,6-DCL-cBIMPS revealed additive inhibitory effect on TRAP-induced P-selectin expression. Taken together, we found no evidence for a stimulatory role of cGMP/PKG in platelets activation and conclude rather that cGMP/PKG signalling has an important inhibitory function in human platelet activation.



## 1. Introduction

Nitric oxide (NO) and prostacyclin ($PGI_2$) modulate platelet function by raising the intracellular concentrations of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), respectively. Elevated levels of these second messengers lead to the activation of cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinases (PKA). The cross-communication between cAMP and cGMP signalling have been reported in platelets [1,2], as well as in the heart [3], smooth muscle [4] and endothelium [5]. In platelets, cross-talk between cAMP and cGMP signalling is mediated mainly through allosteric cGMP stimulation of cAMP degradation by phosphodiesterase 2 (PDE2) and inhibition of the cAMP-hydrolysing activity of PDE3. Thus, cGMP can have both positive and negative effects on the intracellular cAMP levels in platelets. The platelet response to NO and cGMP-induced signalling is in dispute. Several studies show that NO and cGMP-signalling inhibits platelet activation and that the inhibitory actions of NO signalling can be mediated by both PKG-dependent (NO-cGMP-PKG) and PKG-independent pathway (NO-cGMP-PDE3A-cAMP-PKA) [2,6–10]. However, a stimulatory role for NO and cGMP-PKG in low dose agonist-induced platelet activation has also been

*Abbreviations:* 8-Br-PET-cGMP, (β-phenyl-1),$N^2$-etheno-8-bromoguanosine-3',5'-cyclic monophosphate; 8-pCPT-cGMP, *para*-Chlorophenylthioguanosine-3',5'-cyclic monophosphate; Sp-5,6-DCL-cBIMPS, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3',5'-cyclic monophosphorothioate; Sp-isomer, Rp-8-pCPT-cGMPS, 8-(4-Chlorophenylthio)guanosine-3',5'- cyclic monophosphorothioate, Rp- isomer; PKA, cAMP-activated protein kinase; PKG, cGMP-activated protein kinase; TRAP, thrombin receptor agonist peptide.

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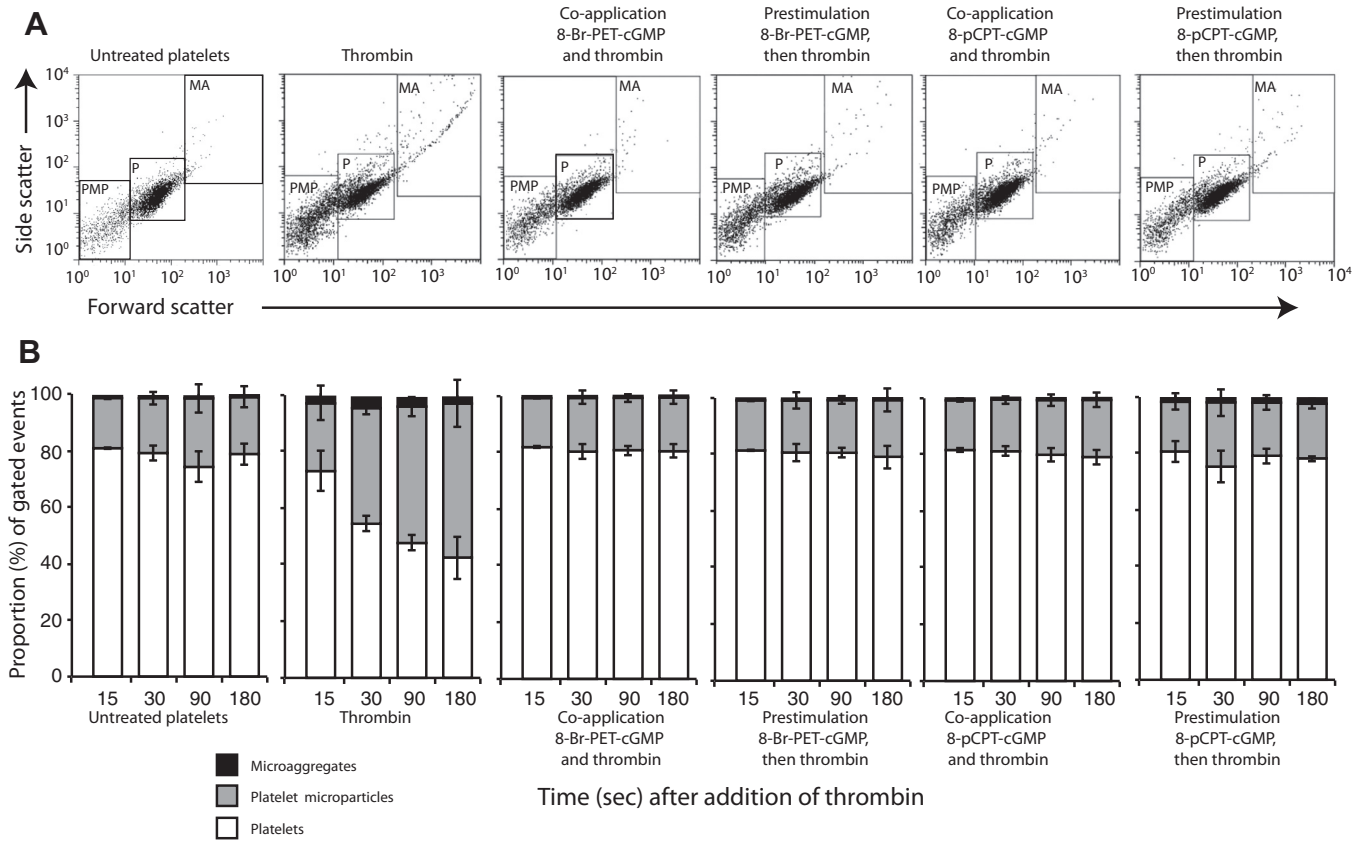



**Fig. 1.** Inhibition of thrombin-induced single platelet disappearance, formation of platelet microparticles and platelets microaggregates after preincubation or co-stimulation with PKG activators. The figure shows flow cytometric data obtained from fixed gel-filtered platelets (GFP) labelled with FITC-conjugated anti-human platelet chicken antibody. Panel (A) shows scatter plots of stirred (900 rpm) platelets treated with thrombin (0.05 U/ml) alone or in combination with the given analogues at 37 °C for 180 s. PMP, P and MA denote the gates for submicron sized platelet-derived microparticles, intact platelet population and microaggregates, respectively. Panel (A) is representative for the data depicted in panel (B), in which data obtained from PMP-, P- and MA-gating of platelets treated for 15, 45, 90 and 180 s are shown. The data show the per cent ratio of platelets (P), platelet microparticles (PMP) and microaggregates (MA) of the gated events illustrated in (A). The data in B represent the mean and SEM of 3–4 separate experiments.

suggested [11–14]. The stimulating effect of cGMP-PKG signalling on washed platelets was observed in the early phase when platelets were costimulated with subthreshold concentrations of agonist (thrombin, von Willebrand factor) and cGMP-elevating drug (sildenafil, NO-donors) or PKG-activating cGMP analogues.

In view of the physiological and medical importance of platelet activation control, it is important to know whether activation of the cGMP pathway and PKG can enhance platelet activation. Possibly, different experimental conditions may explain some of the discrepancies in platelets cGMP-signalling studies. One issue is the choice of PKG activating cGMP analogues, which may have off-target effects [15]. Another issue is the possible side-effects caused by the presence of contaminating cells and/or interfering substances in the platelet preparations. For example, various blood-clotting plasma proteins (e.g., fibrinogen and von Willebrand factor) and ADP from leaky erythrocytes may be present at relatively high concentration in a washed platelet preparation. To isolate functional platelets from plasma, we used a very efficient gel filtration method [16] in all experiments in this study. Moreover, the commonly used method of light transmittance aggrometry is rather insensitive towards single platelet disappearance (SPD) and formation of small platelet aggregates [17,18]. Therefore, in the present study we used flow cytometric assays [19] that allows also the sensitive detection of small platelet aggregates, SPD, shedding of procoagulant platelet-derived microparticles (PMPs) and TRAP-induced P-selectin expression. We found, based on data obtained with a number of cyclic nucleotide analogues, that cGMP/PKG activation consistently inhibited platelet function. No evidence was found supporting any stimulatory or co-stimulatory role of PKG in platelet activation.

## 2. Materials and methods

### 2.1. Materials

Sepharose CL-2B gel matrix was from Pharmacia Biotec (Uppsala, Sweden), and bovine albumin serum (BSA), fraction V (pH 7, lyophilized) was from ICN Biomedicals (Aurora, OH, USA). The synthetic thrombin receptor agonist peptide (TRAP) Ser-Phe-Leu-Leu-Arg-Asn (SFLLRN) from the Biotechnology centre of Oslo (Rikshospitalet, Oslo, Norway) and bovine thrombin (Parke-Davis, Morris Plains, NJ) were diluted in 0.15 M NaCl prior to experiments. The cyclic nucleotide analogues 8-Br-PET-cGMP, 8-pCPT-cGMP and Sp-5,6-DCL-cBIMPS from BioLog (Bremen, Germany) and the nitric oxide donor sodium nitroprusside (SNP) obtained from Sigma–Aldrich (St Louis, MO, USA) were dissolved in water (MQ-quality, Millipore systems Merck-Millipore, Billerica, MA, USA). All other chemicals were from Sigma–Aldrich and of analytical or higher grade.

Antibodies: R-phycoerythrin (R-PE)-conjugated anti-human CD62, a monoclonal antibody directed against P-selectin, was purchased from BDIS (Franklin Lakes, NJ, USA). FITC-conjugated anti-human platelet chicken antibody was from Biopool (Umeå,

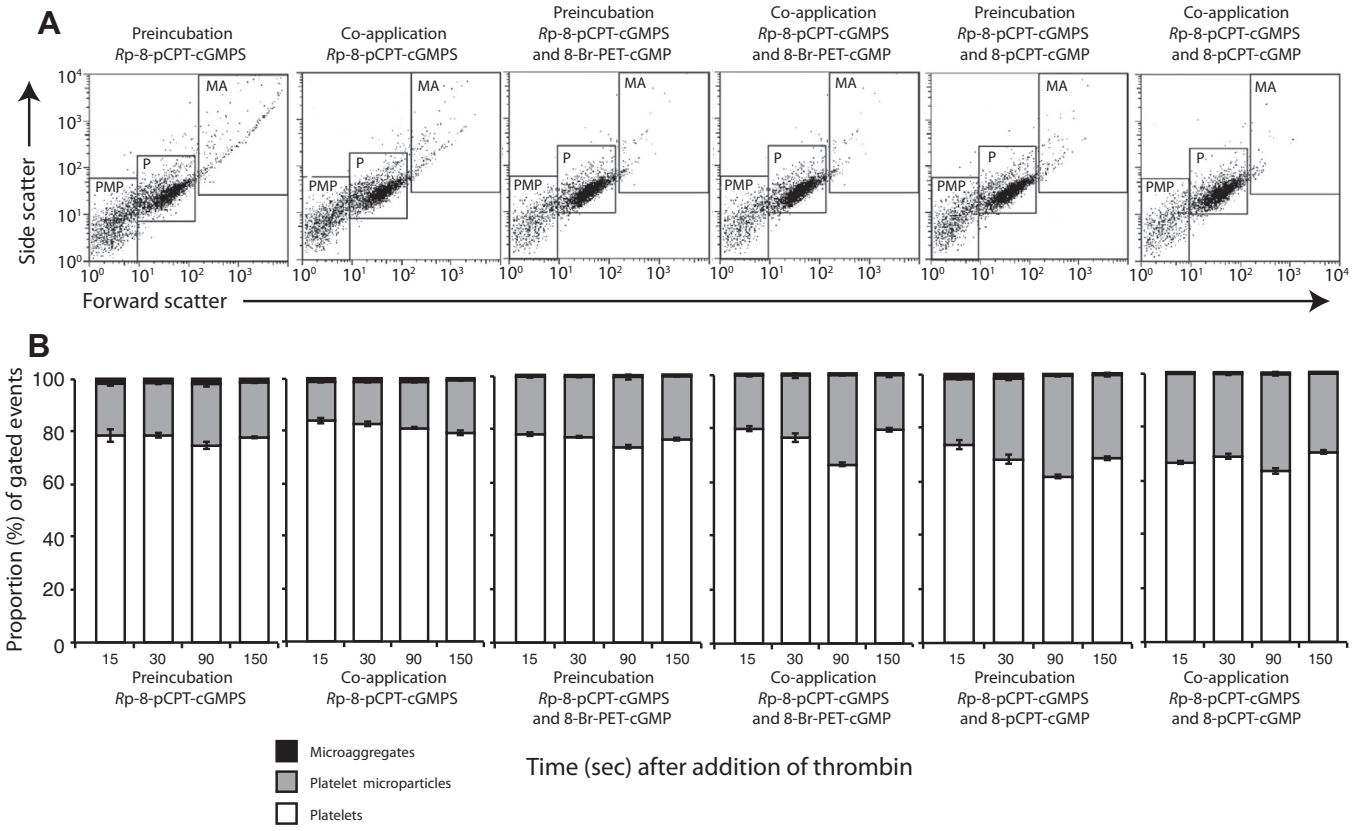



**Fig. 2.** Inhibitory effect of the PKG-antagonist Rp-8-pCPT-cGMPS on thrombin-induced platelets activation. Panel (A) (scatter plots) shows stirred (900 rpm) platelets treated with thrombin (0.05 U/ml) in combination with the PKG activators 8-Br-PET-cGMP (100 μM) or 8-pCPT-cGMP (200 μM) and/or the PKG antagonist Rp-8-pCPT-cGMPS (200 μM) at 37 °C for 180 s. Panel (B) quantitation of the results obtained from gating for PMP-, P- and MA of platelets treated for 15, 45, 90 and 180 s. For data on untreated platelets and platelets treated with thrombin alone, see Fig. 1. Data in B represent the mean ± SEM ($n$ = 3–4).

Sweden). Monoclonal FITC-labelled antibody (mAb) against phosphorylated VASP Ser 157 (5C6) and VASP Ser 259 (16C2) were from Nanotools Antikoerpertechnik (Teningen, Germany).

### 2.2. Isolation and gel filtration of human platelets

Freshly drawn venous blood was provided by the Blood Bank (Haukeland University Hospital, Bergen, Norway). Isolation and gel filtration of human blood platelets was carried out as previously described [16,20]. Gel-filtrated platelet concentration was determined by a ZM Coulter Counter from Coulter Electronics Limited (Luton, UK) and adjusted to $3.5 \times 10^8$ platelets/mL in GFB. Gel-filtrated platelets were equilibrated at RT for at least 30 min prior to experiments.

### 2.3. Assessment of platelet activation

Assessment of P-selectin translocation, platelet microparticles (PMP) formation and single platelet disappearance (SPD) were performed as described elsewhere [19]. Flow cytometric analysis of P-selectin expression, platelet microparticles (PMP) formation and single platelet disappearance (SPD) were detected by a FACSort Flow Cytometer and CellQuest software from Becton Dickinson (San Jose, CA, USA) [19].

### 2.4. Assessment of VASP phosphorylation

Platelets incubated in the absence or presence of SNP or forskolin or a single PKG activator (8-Br-PET-cGMP, 8-pCPT-cGMPS) were analysed for VASP Ser157 and Ser239 phosphorylation by flow cytometry as described elsewhere [21].

### 2.5. Assessment of platelet cAMP content

Platelet cAMP levels in the absence or presence of thrombin and/or PKG activator were determined by a competitive binding assay [21,22].

## 3. Results

### 3.1. Both PKG-activating cGMP analogues and the PKG antagonist Rp-8-pCPT-cGMPS inhibit thrombin-induced platelet activation

Both inhibitory and stimulatory effects of NO and cGMP-PKG signalling have been reported for light transmittance aggregometry studies with washed platelets [12,14,23–28]. Light transmittance aggregometry can be insensitive towards single platelet disappearance (SPD) and formation of small platelet aggregates. Hence, we reasoned that early platelet activation would be more readily noticed using a more sensitive flow cytometric assay able to detect early thrombin-induced formation of small platelet aggregates, SPD, and shedding of procoagulant platelet-derived microparticles (PMP) from the plasma membrane of human gel-filtered platelets.

Our flow cytometric analysis of gel-filtered platelets reproduced the expected [12,23] anti-thrombotic effect of a persistent PKG stimulation. Thus, prolonged preincubation (10 min) with membrane-permeable PKG activators (8-Br-PET-cGMP or 8-pCPT-cGMP) before activation with thrombin (0.05 U/ml), inhibited platelet aggregation/SPD (Fig. 1). The preincubation with PKG activators also inhibited thrombin-induced generation of PMPs and small platelet aggregates (Fig. 1 A and B).

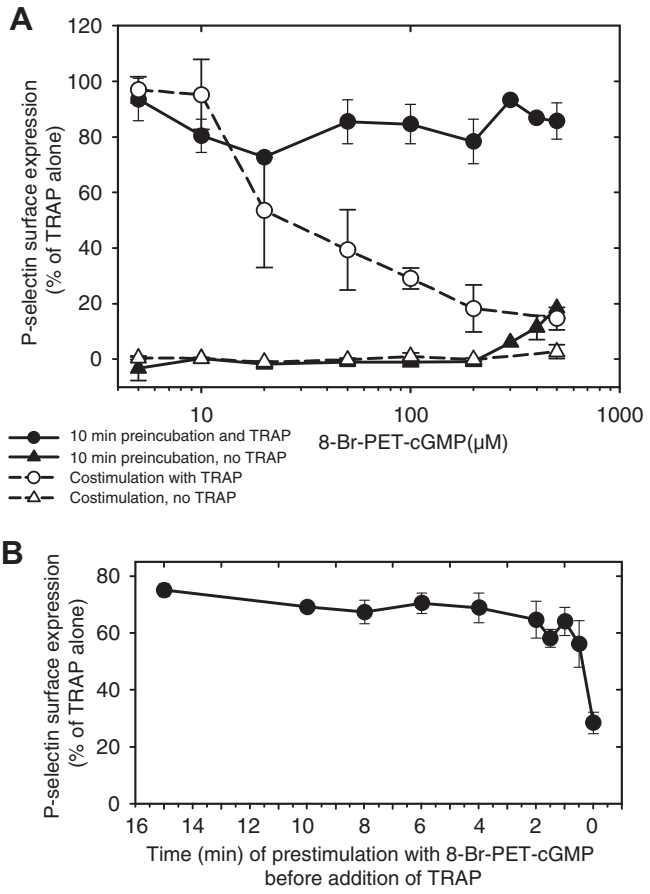



**Fig. 3.** Prolonged incubation time with the PKG activator 8-Br-PET-cGMP reduces its inhibitory effect on TRAP-induced P-selectin expression. The figure shows TRAP-induced surface expression of P-selectin after prestimulation (10 min) or costimulation with different concentrations of the PKG activator 8-Br-PET-cGMP (A). In B, GFP was incubated with 8-Br-PET-cGMP (200 μM) at various time-intervals before addition of TRAP (10 μM). P-selectin surface expression (A and B) was assessed by flow cytometry as described in Section 2. The data are the mean and SEM of 3–6 separate experiments.

We were, however, unable to detect any stimulation in presence of PKG activators at any condition used. Thus, the inhibition of PMP, small platelet aggregates and SPD was equally strong whether the PKG activator (8-Br-PET-cGMP or 8-pCPT-cGMP) was added simultaneously with or 10 min before the low concentration (0.05 U/ml) of thrombin (Fig. 1 A and B).

The notion of an early pro-thrombotic role of PKG is in part based on use of the PKG-antagonist *R*p-8-pCPT-cGMPS [12,13]. We found that *R*p-8-pCPT-cGMPS mimicked the anti-thrombotic action of PKG activators. In fact, it blocked entirely the thrombin-induced SPD and PMP formation, as well as the formation of small platelet aggregates (Fig. 2). The *R*p-8-pCPT-cGMPS analogue acted very rapidly, without need for preincubation (Fig. 2). This suggests that *R*p-8-pCPT-cGMPS can have off-target anti-thrombotic effects, as recently reported for 8-pCPT substituted cAMP analogues on G-protein-coupled receptors [29,30]. In conclusion, we fail to reproduce any pro-thrombotic effect of PKG agonists and reveal that the PKG antagonist *R*p-8-pCPT-cGMPS inhibits aggregation of platelets whose PKG is not stimulated, suggesting off-target effects of the analogue.

### *3.2. Time-dependent inhibitory effect of 8-Br-PET-cGMP on TRAP-induced surface exposure of P-selectin*

We considered next if PKG could stimulate the TRAP-induced platelet surface expression of P-selectin, for these platelets were pretreated (10 min) or costimulated with different concentrations of 8-Br-PET-cGMP before activation with 10 μM TRAP. Preincubation with even high concentrations of 8-Br-PET-cGMP only slightly inhibited P-selectin expression (Fig. 3 A). However, when platelets were costimulated with TRAP (10 μM) and different concentrations of the PKG analogue, it produces an inhibitory effect on α-granule secretion of P-selectin (Fig. 3A). These results initially would suggest that prestimulation of PKG activity has no effect on platelet induced activity, measured as P-selectin surface expression in contrast to the results observed with PMP.

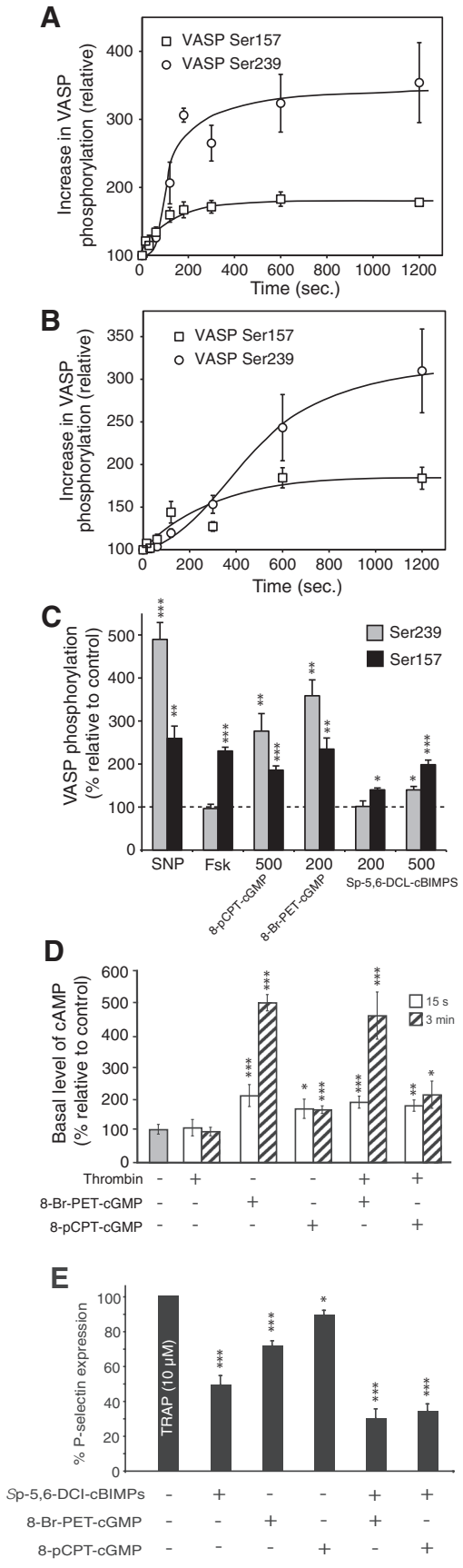
We investigated further this puzzling time-dependence of the inhibitory effect of 8-Br-PET-cGMP. Gel-filtered human platelets were incubated with 8-Br-PET-cGMP (200 μM) at various time-intervals before addition of TRAP (10 μM) (Fig. 3B). Intriguingly, 8-Br-PET-cGMP lost its inhibitory action if preincubated with the platelets for more than 1 min. Thus, for costimulation, the PKG activator 8-Br-PET-cGMP inhibited the TRAP-induced surface expression of P-selectin by 72.5 ± 7.5%, an effect that was reduced to 44.8 ± 14% when 8-Br-PET-cGMP was added 30 s before TRAP. These results suggest that part of the effect of 8-Br-PET-cGMP may be independent of its role as PKG inhibitor since it needs to be present with the TRAP ligand at the moment of receptor activation. Another possibility is that the pretreatment induces some kind of desensitisation response.

### *3.3. Effect of cGMP analogues on VASP phosphorylation and cAMP levels*

We examined whether the time-dependent inhibitory effect of 8-Br-PET-cGMP on TRAP-induced P-selectin surface exposure (Fig. 3A and B) was due to desensitisation of PKG or possible PKA signalling. We measured the temporal changes in site-specific phosphorylation of VASP at Ser239 (mainly PKG) and Ser157 (mainly PKA) in response to the PKG agonists 8-Br-PET-cGMP (200 μM, Fig. 4A) and 8-pCPT-cGMP (Fig. 4B). For either analogue the kinetics of VASP-pSer157 formation was more rapid than for VASP-pSer239. On the other hand, lower steady state stimulation was observed for pSer157 than pSer239. There was no sign of decreased VASP phosphorylation (desensitisation) even after prolonged (>20 min) incubation with either analogue (Fig. 4 A and B).

We compared next the steady state VASP phosphorylation response to stimulation with forskolin and SNP. As expected, we found that forskolin and the PKA agonist Sp-5,6-DCL-cBIMPS promoted phosphorylation of VASP at the Ser157 site (PKA-mediated) and that the NO-donor SNP and 8-Br-PET-cGMP induced phosphorylation at both the Ser239 and the Ser157 sites [21] (Fig. 4C). Importantly, we found no evidence for desensitisation of PKG or PKA activity, measured as site-specific VASP phosphorylation, after sustained incubation with one of the two PKG analogues or through stimulation of the cyclases (Fig. 4 C).

We conclude that the PKG activating analogues used produced the expected VASP phosphorylation state changes, and that their apparently anomalous lack of effect on P-selectin surface expression during long term experiments did not coincide with deficient phosphorylation of the PKG responsive site Ser239 of VASP.




### 3.4. PKG activators increase the levels of cAMP in platelets

The rapid VASP Ser157 phosphorylation in the presence of the PKG-activator 8-Br-PET-cGMP occurred with a time kinetic similar to that of the loss of the 8-Br-PET-cGMP ability to inhibit P-selectin externalisation. We considered therefore whether 8-Br-PET-cGMP could activate platelet PKA. One mechanism could be through increase of cAMP, through inhibition of cAMP-PDEs like PDE2A and/or PDE3A, which control the levels of platelet cAMP. We therefore examined whether prolonged incubation with PKG analogues had an effect on the intracellular levels of cAMP in platelets (Fig. 4D). We found that 8-Br-PET-cGMP rapidly (15 s) doubled the level of cAMP in platelets, and after 3 min incubation with 8-Br-PET-cGMP the intracellular basal cAMP levels in platelets had increased by approximately 400%. We observed only moderately elevated cAMP levels when platelets were incubated with the potent PKG agonist 8-pCPT-cGMP (Fig. 4D). These findings explain the much more rapid phosphorylation of Ser157 of VASP in response to 8-Br-PET-cGMP as compared to 8-pCPT-cGMP (Fig. 4A). They indicate also that PKA activation occurs in platelets preincubated with 8-Br-PET-cGMP.

### 3.5. Additive inhibitory effect between PKA and PKG activators on α-granule exposure of P-selectin

P-selectin expression has been suggested to be differentially regulated by PKA and PKG [31]. When platelet were preincubated with different concentrations of the PKA-activator Sp-5,6-DCL-cBIMPS (10–200 μM) for 10 min before stimulation with TRAP (10 μM), the IC50 for inhibition of α-granule secretion of P-selectin was found to be 50 μM. To examine possible cross-communications between PKA and PKG on α-granule secretory pathways, we simultaneously added TRAP (10 μM) and PKG (8-Br-PET-cGMP or 8-pCPT-cGMP) activator 10 min after addition of the PKA activator Sp-5,6-DCL-cBIMPS. A clear additive inhibitory effect between PKA and PKG activators on TRAP-induced expression of P-selectin was observed under these conditions (Fig. 4E).

## 4. Discussion

In the present study we investigated the effect of PKG agonists on platelet activation using gel-filtered human platelets to exclude possible influence of plasma proteins (e.g., fibrinogen and von Willebrand factor) and ADP from leaky erythrocytes, which may

**Fig. 4.** Temporal and steady state phosphorylation of VASP are shown in A–C. The temporal change in platelet VASP Ser157 and Ser239 phosphorylation is shown in response to (A) 8-Br-PET-cGMP (200 μM) and (B) 8-pCPT-cGMP (500 μM). (C) shows the steady state increase in VASP Ser157 and Ser239 phosphorylation in response to SNP (100 μM), forskolin (Fsk, 5 μM), 8-pCPT-cGMP, 8-Br-PET-cGMP and Sp-5,6-DCL-cBIMPS. The numbers below the bars denote analogue concentration (μM). Panels A–C show VASP phosphorylation relative to control (100%) as measured by flow cytometry. (D) shows changes in the platelets cAMP level after incubation with 8-Br-PET-cGMP (100 μM) or 8-pCPT-cGMP (200 μM) in the absence or presence of thrombin (0.1 U/ml). (E) shows the additive inhibitory effect between the PKA activator Sp-5,6-DCL-cBIMPS (50 μM) and PKG activators (20 μM 8-Br-PET-cGMP or 50 μM 8-pCPT-cGMP) on TRAP-induced P-selectin expression. The data (A–E) are representative for 3–5 separate experiments, mean ± SEM ($n$ = 3–20). The asterisks indicate significance level of $p < 0.05$ (*), 0.01(**) or 0.005 (***), Student $t$-test where the means are tested for significant difference from non-stimulated platelets (C and D), or TRAP-stimulated platelets (E).

be present at relative high concentration in washed platelet preparation. We show that activators of PKG consistently inhibit platelet activation without evidence for any initial costimulatory effect at subthreshold concentrations of thrombin or TRAP. This is in contrast with previous reports by Li et al. [12,13] using visible light based aggregometry and washed platelets. Our finding was consistent whether platelet activation was judged by SPD, PMP formation or platelet aggregation. We also found that the PKG-antagonist *Rp*-8-pCPT-cGMPS inhibits platelet activation. The inhibitory actions of this and similar PKG inhibitors have been interpreted as an important proof of a stimulatory action of cGMP/PKG signalling in platelets [12,27]. However, the inhibitory actions of the PKG-antagonists *Rp*-8-pCPT-cGMPS and *Rp*-8-Br-PET-cGMPS, were proven to be mediated via a non-PKG effect by two other groups [23,24]. In particular, Marshall et al. demonstrated that PKG inhibitors like Rp-8-pCPT-cGMPS also inhibited platelet activation in PKG-deficient platelets [24].

It has also been reported that PKG has an opposing effect on PKA-mediated inhibition of thrombin-induced P-selectin expression when cAMP levels are elevated [31]. However, these findings were based on the use of the unspecific PKA and PKG inhibitors H89 and KT5823, respectively. H89 inhibits PKA and PKG, as well as many other protein kinases, and KT5823 fails to inhibit PKG-induced signalling in intact platelets [32–34]. Here, we demonstrate an additive inhibitory effect between the PKA activator Sp-5,6-DCL-cBIMPS and PKG activators (8-Br-PET-cGMP or 8-pCPT-cGMP) on TRAP-induced P-selectin expression (Fig. 4E). Interestingly, prolonged incubation time with the 8-Br-PET-cGMP analogue reduced its inhibition of TRAP-induced P-selectin expression through a mechanism that does not appear to involve desensitisation of PKA or PKG activity. This was surprising as it increased total cAMP levels in platelets, particularly during long time incubations. However, this is still consistent with observations that inhibition of PDE2A give substantial increase in cAMP levels, but are largely inefficient in inhibiting P-selectin exposure and integrin αIIbβ3 activation in response to TRAP [1,35]. Compared to the efficiency of PDE3A inhibitor this is still not resolved in platelets, but strongly suggests the presence of at least two cAMP-responsive compartments with different regulation of platelet activation.

We here suggest that part of the effects of the PKG activators \8-Br-PET-cGMP and 8-pCPT-cGMP, as well as the PKG-antagonists Rp-8-pCPT-cGMPS, are off-target/non-PKG effect on plasma membrane receptors, as recently reported for 8-pCPT substituted cAMP analogues on G-protein-coupled receptors [29,30]. All in all, we found no evidence for a stimulatory role of cGMP/PKG in platelets activation and conclude that cGMP/PKG signalling has an important inhibitory function in human platelet activation.

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## References

[1] T.R. Dunkern, A. Hatzelmann, The effect of Sildenafil on human platelet secretory function is controlled by a complex interplay between phosphodiesterases 2, 3 and 5, Cell. Signal. 17 (2005) 331–339.
[2] B.O. Jensen, F. Selheim, S.O. Doskeland, A.R.L. Gear, H. Holmsen, Protein kinase A mediates inhibition of the thrombin-induced platelet shape change by nitric oxide, Blood 104 (2004) 2775–2782.
[3] M. Zaccolo, M.A. Movsesian, CAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology, Circ. Res. 100 (2007) 1569–1578.
[4] R. Worner, R. Lukowski, F. Hofmann, J.W. Wegener, CGMP signals mainly through cAMP kinase in permeabilized murine aorta, Am. J. Physiol. Heart. Circ. Physiol. 292 (2007) H237–244.
[5] P. He, M. Zeng, F.E. Curry, Dominant role of cAMP in regulation of microvessel permeability, Am. J. Physiol. Heart. Circ. Physiol. 278 (2000) H1124–1133.
[6] U.R. Schwarz, U. Walter, M. Eigenthaler, Taming platelets with cyclic nucleotides, Biochem. Pharm. 62 (2001) 1153–1161.
[7] M. Antl, M.-L. von Bruehl, C. Eiglsperger, M. Werner, I. Konrad, T. Kocher, M. Wilm, F. Hofmann, S. Massberg, J. Schlossmann, IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation and thrombus formation, Blood 109 (2007) 552–559.
[8] R.J. Haslam, N.T. Dickinson, E.K. Jang, Cyclic nucleotides and phosphodiesterases in platelets, Thromb. Haemostasis 82 (1999) 412–423.
[9] D.E. Knight, M.C. Scrutton, Cyclic-nucleotides control a system which regulates Ca-2+ sensitivity of platelet secretion, Nature 309 (1984) 66–68.
[10] Z.Y. Li, J. Ajdic, M. Eigenthaler, X.P. Du, A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans, Blood 101 (2003) 4423–4429.
[11] P.F. Blackmore, Biphasic effects of nitric oxide on calcium influx in human platelets, Thromb. Res. 127 (2011) E8–E14.
[12] Z.Y. Li, X.D. Xi, M.Y. Gu, R. Feil, R.D. Ye, M. Eigenthaler, F. Hofmann, X.P. Du, A stimulatory role for cGMP-dependent protein kinase in platelet activation, Cell 112 (2003) 77–86.
[13] Z.Y. Li, G.Y. Zhang, J.A. Marjanovic, C.G. Ruan, X.P. Du, A platelet secretion pathway mediated by cGMP-dependent protein kinase, J. Biol. Chem. 279 (2004) 42469–42475.
[14] G. Zhang, B. Xiang, A. Dong, R.C. Skoda, A. Daugherty, S.S. Smyth, X. Du, Z. Li, Biphasic roles for soluble guanylyl cyclase (sGC) in platelet activation, Blood 118 (2011) 3670–3679.
[15] H. Poppe, S.D. Rybalkin, H. Rehmann, T.R. Hinds, X.-B. Tang, A.E. Christensen, F. Schwede, H.-G. Genieser, J.L. Bos, S.O. Doskeland, J.A. Beavo, E. Butt, Cyclic nucleotide analogs as probes of signaling pathways, Nat. Met. 5 (2008) 277–278.
[16] O.B. Tysnes, G.M. Aarbakke, A.J.M. Verhoeven, H. Holmsen, Thin-layer chromatography of polyphosphoinositides from platelet extracts – interference by an unknown phospholipid, Thromb. Res. 40 (1985) 329–338.
[17] G.V.R. Born, Aggregation of blood platelets by adenosine diphosphate and its reversal, Nature 194 (1962) 927.
[18] G.V. Born, M.J. Cross, The aggregation of blood platelets, J. Physiol. 168 (1963) 178–195.
[19] F. Selheim, H. Holmsen, F.S. Vassbotn, Platelet-derived growth factor inhibits platelet activation in heparinized whole blood, Thromb. Res. 95 (1999) 185–196.
[20] F. Selheim, A.K. Froyset, I. Strand, F.S. Vassbotn, H. Holmsen, Adrenaline potentiates PI 3-kinase in platelets stimulated with thrombin and SFRLLN: role of secreted ADP, FEBS Lett. 485 (2000) 62–66.
[21] B.O. Jensen, R. Kleppe, R. Kopperud, G. Nygaard, S.O. Doskeland, H. Holmsen, F. Selheim, Dipyridamole synergizes with nitric oxide to prolong inhibition of thrombin-induced platelet shape change, Platelets 22 (2011) 8–19.
[22] S.O. Doskeland, H.J. Haga, Measurement of adenosine 3'-5'-cyclic monophosphate by competitive binding to salt-dissociated protein-kinase, Biochem. J. 174 (1978) 363–372.
[23] S. Gambaryan, J. Geiger, U.R. Schwarz, E. Butt, A. Begonja, A. Obergfell, U. Walter, Potent inhibition of human platelets by cGMP analogs independent of cGMP-dependent protein kinase, Blood 103 (2004) 2593–2600.
[24] S.J. Marshall, Y.A. Senis, J.M. Auger, R. Feil, F. Hofmann, G. Salmon, J.T. Peterson, F. Burslem, S.P. Watson, GPIb-dependent platelet activation is dependent on Src kinases but not MAP kinase or cGMP-dependent kinase, Blood 103 (2004) 2601–2609.
[25] J.A. Marjanovic, Z.Y. Li, A. Stojanovic, X.P. Du, Stimulatory roles of nitric-oxide synthase 3 and guanylyl cyclase in platelet activation, J. Biol. Chem. 280 (2005) 37430–37438.
[26] S. Gambaryan, A. Friebe, U. Walter, Does the NO/sGC/cGMP/PKG pathway play a stimulatory role in platelets?, Blood 119 (2012) 5335–5336
[27] Z.Y. Li, G.Y. Zhang, R. Feil, J.H. Han, X.P. Du, Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin alpha(IIb)beta(3), Blood 107 (2006) 965–972.
[28] A.J. Begonja, J. Geiger, N. Rukoyatkina, S. Rauchfuss, S. Gambaryan, U. Walter, Tbrombin stimulation of p38 MAP kinase in human platelets is mediated by ADP and thromboxane A(2) and inhibited by cGMP/cGNW-dependent protein kinase, Blood 109 (2007) 616–618.
[29] L. Herfindal, G. Nygaard, R. Kopperud, C. Krakstad, S.O. Doskeland, F. Selheim, Off-target effect of the Epac agonist 8-pCPT-2 '-O-Me-cAMP on P2Y(12) receptors in blood platelets, Biochem. Biophys. Res. Commun. 437 (2013) 603–608.
[30] C. Sand, M. Grandoch, C. Boergermann, P.A.O. Weernink, Y. Mahlke, B. Schwindenhammer, A.-A. Weber, J.W. Fischer, K.H. Jakobs, M. Schmidt, 8-pCPT-conjugated cyclic AMP analogs exert thromboxane receptor antagonistic properties, Thromb. Haemostasis 103 (2010) 662–676.
[31] D. Libersan, G. Rousseau, Y. Merhi, Differential regulation of P-selectin expression by protein kinase A and protein kinase G in thrombin-stimulated human platelets, Thromb. Haemostasis 89 (2003) 310–317.
[32] M. Burkhardt, M. Glazova, S. Gambaryan, T. Vollkommer, E. Butt, B. Bader, K. Heermeier, T.M. Lincoln, U. Walter, A. Palmetshofer, KT5823 inhibits cGMP-

dependent protein kinase activity in vitro but not in intact human platelets and rat mesangial cells, J. Biol. Chem. 275 (2000) 33536–33541.
[33] R. Kleppe, C. Krakstad, F. Selheim, R. Kopperud, S.O. Doskeland, The cAMP-dependent protein kinase pathway as therapeutic target – possibilities and pitfalls, Curr. Top. Med. Chem. 11 (2011) 1393–1405.
[34] J. Bain, H. McLauchlan, M. Elliott, P. Cohen, The specificities of protein kinase inhibitors: an update, Biochem. J. 371 (2003) 199–204.
[35] M.A.H. Feijge, K. Ansink, K. Vanschoonbeek, J.W.M. Heemskerk, Control of platelet activation by cyclic AMP turnover and cyclic nucleotide phosphodiesterase type-3, Biochem. Pharm. 67 (2004) 1559–1567.