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Off-target effect of the Epac agonist 8-pCPT-2′-O-Me-cAMP on $P2Y_{12}$ receptors in blood platelets

Lars Herfindal [a,b,1], Gyrid Nygaard [a,c,1], Reidun Kopperud [a], Camilla Krakstad [a,2], Stein Ove Døskeland [a], Frode Selheim [a,c,*]

[a] Department of Biomedicine, University of Bergen, Bergen, Norway
[b] Translational Signaling Group, Haukeland University Hospital, N-5009 Bergen, Norway
[c] Proteomics Unit at University of Bergen (PROBE), University of Bergen, Bergen, Norway

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ABSTRACT

The primary target of the cAMP analogue 8-pCPT-2′-O-Me-cAMP is exchange protein directly activated by cAMP (Epac). Here we tested potential off-target effects of the Epac activator on blood platelet activation signalling. We found that the Epac analogue 8-pCPT-2′-O-Me-cAMP inhibits agonist-induced-GPCR-stimulated, but not collagen-stimulated, P-selectin surface expression on Epac1 deficient platelets. In human platelets, 8-pCPT-2′-O-Me-cAMP inhibited P-selectin expression elicited by the PKC activator PMA. This effect was abolished in the presence of the extracellular ADP scavenger system CP/CPK. *In silico* modelling of 8-pCPT-2′O-Me-cAMP binding into the purinergic platelet receptor $P2Y_{12}$ revealed that the analogue docks similar to the $P2Y_{12}$ antagonist 2MeSAMP. The 8-pCPT-2′-O-Me-cAMP analogue *per se*, did not provoke Rap 1 (Rap 1-GTP) activation or phosphorylation on the vasodilator-stimulated phosphoprotein (VASP) at Ser-157. In addition, the protein kinase A (PKA) antagonists Rp-cAMPS and Rp-8-Br-cAMPS failed to block the inhibitory effect of 8-pCPT-2′-O-Me-cAMP on thrombin- and TRAP-induced Rap 1 activation, thus suggesting that PKA is not involved. We conclude that the 8-pCPT-2′-O-Me-cAMP analogue is able to inhibit agonist-induced-GPCR-stimulated P-selectin independent from Epac1; the off-target effect of the analogue appears to be mediated by antagonistic $P2Y_{12}$ receptor binding. This has implications when using cAMP analogues on specialised system involving such receptors. We found, however that the Epac agonist 8-Br-2′-O-Me-cAMP did not affect platelet activation at similar concentrations.

1. Introduction

Cyclic adenosine monophosphate (cAMP) is a key regulator in cell signalling, and cAMP analogues have been produced to ensure selectivity towards subunits of PKA and Epac, as well as to enhance membrane permeability, or to prevent degradation by phosphodiesterases [1–4]. cAMP analogues are invaluable tools to study the role of PKA and Epac in cell biology. However, the specificity of small agonists/antagonists has been questioned, apparent in several extensive studies on protein kinases [5–7]. To avoid misinterpretations of the data obtained by molecules targeted to one ligand, it is important to map off-target effects.

We have previously demonstrated that 8-pCPT-2′-O-Me-cAMP is a strong Epac1 activator [3]. However, the 8-pCPT moiety of this Epac agonist has been reported to have thromboxane A_2 (TxA_2) receptor antagonistic properties [8]. TxA_2 agonist-induced platelet activation is dependent on positive feedback from secreted ADP [9,10].

When platelets are stimulated by a strong agonist a sequence of platelet responses are initiated [11], this includes synthesis (e.g.,

Abbreviations: 8-Br-PET-cGMP, (β-phenyl-1), N^2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphate; 8-pCPT-2′-O-Me-cAMP, 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate; 2MeSADP, 2-methylthio-adenosine diphosphate; 2MeSAMP, 2-methylthio-adenosine monophosphate; ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; Rp-cAMPS, (Rp)-adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer; Rp-8-Br-cAMPS, 8-bromoadenosineadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer; Sp-5, 6-DCL-cBIMPS, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-cyclic monophosphorothioate, Sp-isomer; Sp-8-pCPT-2′-O-Me-cAMPS, 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer; CP/CPK, creatine phosphate/creatine phosphokinase; Epac, exchange factor directly activated by cAMP; PI3 K, phosphatidyl-inositol-3 kinase; PKA, cAMP-activated protein kinase; PKG, cGMP-activated protein kinase; TxA_2, thromboxane receptor A_2; PMA, phorbol 12-myristate 13-acetate.

* Corresponding author. Address: Department of Biomedicine, University of Bergen, Jonas Lies vei 91, N-5009 Bergen, Norway. Fax: +47 55586360.
E-mail address: Frode.Selheim@biomed.uib.no (F. Selheim).

[1] These authors contributed equally to this study.
[2] Present address: Department of Clinical Science, University of Bergen, Norway.

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TxA_2 and PAF) and secretion of secondary agonists from platelets granules (e.g., ADP from dense granules and fibrinogen and growth factors from α-granules). The secreted agonists additively or synergistically increase the power of the primary agonist. Increased tyrosine phosphorylation and activation of PI3K by thrombin via G-protein coupled receptor (GPCR), but not collagen, are dependent on positive feedback, mainly by secreted ADP [12–14]. The ADP effect can be blocked by addition of CP/CPK [12], which converts ADP to ATP. PMA, a PKC activator also utilise ADP as autocrine signal, and activation by PMA is also inhibited by CP/CPK [10].

Here we suggest an off-target effect of 8-pCPT-2′-O-Me-cAMP on G-protein-coupled receptors in platelets. We demonstrate that 8-pCPT-2′-O-Me-cAMP inhibits agonist-induced-GPCR-stimulated P-selectin expression in Epac1 deficient platelets, and that the Epac agonist appears to act as a $P2Y_{12}$ receptor antagonist. The importance of synergistic positive feedback loop by secreted ADP and TxA_2 are well documented for TRAP- and thrombin-induced platelet signalling [12–17], and ADP signalling via G_{i2}-coupled $P2Y_{12}$ receptors is essential for TxA_2-induced platelet activation via G_q/G_{13} coupled receptors, and vice versa [17,18]. Our data are consistent with such a scenario, indicating that the 8-pCPT-2′-O-Me-cAMP analogue can act antagonise not only TxA_2 [8] but also the purinergic receptor $P2Y_{12}$.

2. Materials and methods

2.1. Materials

All cyclic nucleotide analogues (see abbreviation list for full names) were from BioLog (Bremen, Germany), and were dissolved in PBS (136.7 mM NaCl, 2.7 mM KCl, 13.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). Thrombin (Parke Davis, Morris Plains, NJ) and the synthetic thrombin receptor agonist peptide (TRAP) Ser-Phe-Leu-Leu-Arg-Asn (SFLLRN) from the Biotechnology centre of Oslo (Rikshospitalet, Oslo, Norway) were dissolved in 0.15 NaCl. Type I collagen (Vitrogen 100, 97% Type I and 3% Type III collagen) was from Angiotech BioMaterials (Palo Alto, CA). All other chemicals were from Sigma–Aldrich (St. Louis, MO). Antibodies: R-phycoerythrin (R-PE)-conjugated anti-human CD62 was from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA). PE Rat Anti-Mouse CD41 and FITC RAT Anti-Mouse CD62P were from BD Biosciences (Franklin Lakes, NJ). Monoclonal unlabelled and FITC-labelled antibodies (mAb) against phosphorylated VASP Ser 157 (5C6) and VASP Ser 259 (16C2) were from Nanotools Antikoerpertechnik (Teningen, Germany). Rap1 antibody was from Santa Cruz Biotechnology. Horseradish peroxidase conjugated anti-rabbit IgG was from Transduction Laboratories (Lexington, KY).

2.2. Experimental animals

Epac1$^{-/-}$ mouse [19] was generated by deletion of the cAMP-binding domain by excision of exon 7–10, confirmed by genotyping of ear lobe biopsies. The mice were bred against a C57BL/6JBomTac (Taconic) genetic background. The animal experiments were approved by the Norwegian Animal Research Authority and conducted according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes. The mice were housed in a pathogen-free facility with artificial lighting on a 12:12 h light–dark cycle and temperature was 23 °C. The mice and had access to water and chow ad libitum.

2.3. Isolation of mouse platelets

Blood was obtained from mice euthanized with CO_2. Between 750 and 1000 μl was drawn from the left ventricle into a 2 ml syringe containing 100 μl ACD and 200 μl Ca^{2+}-free Tyrode's buffer. The blood was centrifuged at 300*g* for 5 min, and the resulting platelet-rich plasma (PRP) further centrifuged at 1000*g* for 10 min in the presence of 10 μl acid citrate dextrose (ACD; 71 mM citric acid, 85 mM Na_3-citrate, 100 mM glucose). The plasma supernatant was transferred to Eppendorf tubes and the pelleted platelets resuspended in Tyrode's buffer (136 mM NaCl, 2.7 mM KCl, 0.77 mM $NaH_2PO_4 \times 2H_2O$, and 2.0 mM $MgCl_2 \times 6H_2O$, pH 7.3) containing 5 mM glucose and 0.05% BSA [12], and adjusted to 2.5×10^8 platelets/ml. Platelets were allowed to rest for 30 min at RT before use.

2.4. Isolation of human platelets

Freshly drawn venous blood was obtained from healthy donors at the blood bank, Haukeland University Hospital, Bergen, Norway. The blood was collected into 0.15 volume of ACD. PRP was isolated by centrifugation for 6 min at 535*g* at RT. The platelets in the PRP were pelleted by centrifugation at 1200*g* for 10 min and resuspended in one-third volume of autologous plasma. The platelets were isolated from plasma by size-exclusion through a Sepharose CL-2B gel (Pharmacia Biotec, Sweden) [20]. Platelet numbers were adjusted to a concentration of 3.5×10^8 platelets/ml in Ca^{2+}-free Tyrode's buffer.

2.5. Assessment of P-selectin translocation

Gel filtered human platelets (GFP, 5 μl) were added to polystyrene tubes containing R-PE-conjugated anti-human CD62 (5 μl) and cyclic nucleotide analogues or vehicles in PBS (40 μl). After 5 min incubation, the samples were stimulated with 20 μM TRAP for 20 min without stirring and fixed with 0.2% paraformaldehyde in PBS. Flow cytometric detection of P-selectin expression was by a FACSort Flow Cytometer and CellQuest software (BDIS) [21]. Mouse platelets (5 μl) were added to polystyrene tubes containing FITC-conjugated anti-mouse CD62P and PE-conjugated Anti-Mouse CD41, 8-pCPT-2′O-Me-cAMP (0.3 μM) and/or agonists. The samples (50 μl in PBS) were incubated for 15 min at RT without stirring before fixation in 0.2% paraformaldehyde and the level of P-selectin externalization was assessed by flow cytometry using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ) and FlowJo Software (ver. 9.4.11, Tree Star Inc, Ashland, OR). Five thousand particles identified as platelets by CD41 and forward- and side-scatter distributions were analysed for binding of anti-CD62-FITC per sample.

2.6. Assessment of VASP phosphorylation

Platelets incubated in the absence or presence of cyclic nucleotide analogues were analysed for VASP phosphorylation. Flow cytometric assay to assess VASP phosphorylation was performed with FITC-labelled mAb against phosphorylated P-Ser157 (mAb-5C6) or P-Ser239 (mAb-16C2) as described [22]. Ten thousand particles identified as platelets by forward and side scatter distribution were analysed for FITC-fluorescence, detected with a FL1 channel and a photomultiplier tube voltage of 650 V. Western blotting using primary mAb-5C6 or mAb-16C2 antibodies and secondary horseradish peroxidase conjugated anti-rabbit IgG antibody were described [20].

2.7. Rap 1 activation assay

Rap1-GTP was captured and pulled-down with the Ral-GDS-RBD-GST fusion protein before Western blot detection with specific Rap1 antibody [23].

2.8. In silico modeling of the binding of 8-pCPT-2′O-Me-cAMP, 2MeSAMP, and 2MeSADP

The model of $P2Y_{12}$ liganded with 2MeSAMP was kindly provided by Prof. Kenneth A. Jacobson (Laboratory of Bioorganic Chemistry and Molecular Recognition Section, NIH, Bethesda, MD, USA), and has been published elsewhere [24]. Docking of agonists and antagonists were done in the Maestro software (ver. 9.2; Schrödinger, LLC: New York, NY, USA, 2011). Low energy conformations of ligand tautomers at neutral pH were generated (force field: OPLS_2005, solvation treatment: distance dependent dielectric, max. rel. energy diff: 10 kcal/mol, RMSD of 1.0 Å). The minimized conformations of each ligand were used as starting point for the docking performed by the Glide application of the Maestro software package using extra precision (XP) setting. Visualization of the structures was by Discovery Studio Modeling Environment, (rel. 3.5; Accelrys Software Inc.: San Diego, CA, USA, 2012).

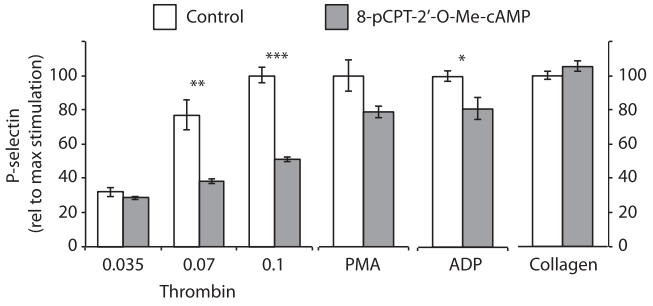

Fig. 1. Inhibition of platelets activation by 8-pCPT-2′-O-Me-cAMP is not connected to Epac1. Blood platelets from Epac1$^{-/-}$ mice were treated with 8-pCPT-2′O-Me-cAMP (0.3 μM) or vehicle for 5 min before addition of the indicated activators for 15 min. Assessment of P-selectin expression was done by flow cytometry as described in the Methods section. The data are mean of 3–6 experiments and s.e.m. The asterisks indicate significance at $p < 0.05$ (∗), $p < 0.01$ (∗∗) or $p < 0.005$ (∗∗∗), *t*-test.

3. Results and discussion

3.1. The Epac-agonist 8-pCPT-2′-O-Me-cAMP inhibits GPCR-stimulated P-selectin expression in Epac1 deficient platelets

During our previous research on blood platelet signalling, we observed that various cAMP analogues could cause platelet inhibition [1]. mRNA for Epac1 [25], but not for Epac2 [26], has been detected, but only trace amounts of Epac1 protein was found [25]. To conclude whether 8-pCPT-2′-O-Me-cAMP inhibited platelets in an Epac-independent manner, we incubated platelets from Epac1 deficient mice with 8-pCPT-2′-O-Me-cAMP for 5 min before activation (Fig. 1). This Epac agonist inhibited thrombin-, PMA- and ADP-induced surface expression of P-selectin in Epac$^{-/-}$ platelets, but failed to inhibit collagen-induced activation. In contrast to ADP, thromboxane A_2 (TxA_2) and thrombin that acts through G-protein-coupled receptors (GPCR) [27], collagen is acting through other receptor types such as the tyrosine kinase-linked GPVI [28]. We conclude that 8-pCPT-2′-O-Me-cAMP inhibits platelet activation independent from Epac; the off-target effect of the analogue appears to be via GPCR signalling.

3.2. Inhibition of agonist-induced P-selectin expression by 8-pCPT-2′-O-Me-cAMP is dependent on secreted ADP

The cAMP analogue 8-pCPT-2′-O-Me-cAMP is a strong Epac1 agonist with low affinity for PKA [3]. However, recent observation suggests that 8-pCPT substituted cAMP-analogues act as thromboxane A_2 (TxA_2) receptor antagonist, reducing thromboxane

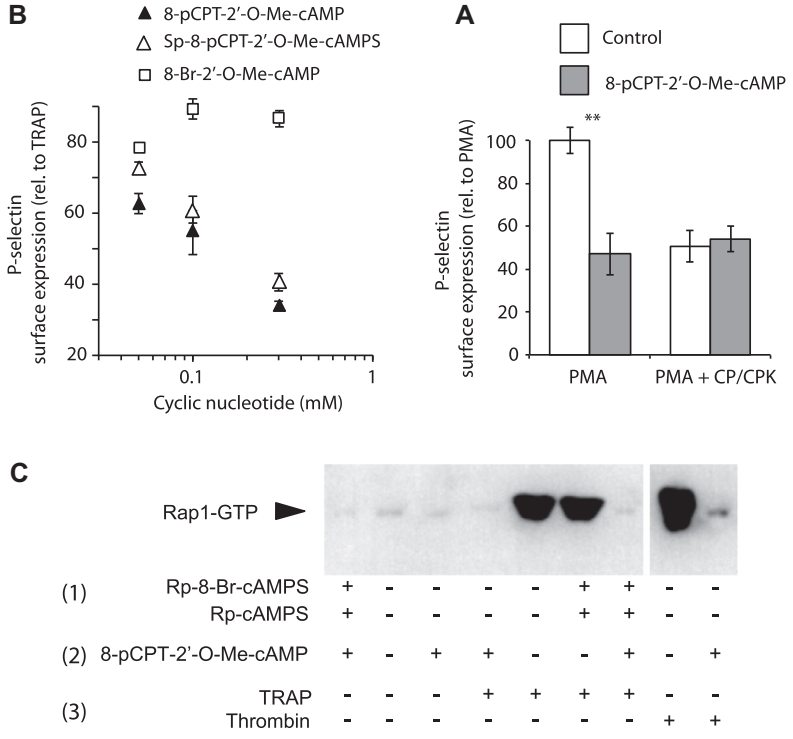

Fig. 2. 8-pCPT substituted cAMP-analogue Epac-agonists inhibit platelet activation, whereas 8-Br-does not. (A) Isolated human platelets were treated with increasing concentrations of analogues for 5 min before addition of 20 μM TRAP. The externalization of P-selectin was assessed by flow cytometry as described in the Methods section. (B) Platelets were treated with 8-pCPT-2′-O-Me-cAMP (1 mM) or vehicle for 10 min before activation by 1 μM phorbol myristate acetate (PMA) with or without the presence of CP/CPK and P-selectin externalization measured as in A. C: Platelets were treated with (1) Rp-analogues (0.5 mM) for 3 min to inhibit PKA before incubation with (2) 8-pCPT-2′-O-Me-cAMP (2 mM) for another 3 min. Platelets were then activated with (3) 20 μM TRAP or 0.1 U/ml of Thrombin for 3 min. Phosphorylation of Rap1-GTP was visualized by western blotting. The data in A and B are average of 3–5 experiments and s.e.m. The asterisks indicate significance at $p < 0.01$ (*t*-test).

agonist U46619-induced contractile response in thoracic aortic rings from rats, and platelet aggregation in human platelets [8]. Interestingly, U46619 does not promote platelet aggregation in the absence of ADP [9]. We failed to inhibit thrombin-induced platelet shape change with 8-pCPT-2′-O-Me-cAMP in the presence of both acetylsalicylic acid (ASA) [inhibits cox-1 and thereby blocks synthesis of TxA_2] and CP/CPK [converts ADP to ATP] (see [20]). In view of these results we wanted to explore whether Epac activators affected on platelet activation. The 8-pCPT-conjugated Epac agonists inhibited TRAP-induced surface expression of P-selectin (Fig. 2A) and thrombin-induced platelet aggregation in ASA treated platelets (data not shown), whereas the Epac agonist 8-Br-2′-O-Me-cAMP showed minimal inhibition at doses up to 0.3 mM. The effect of 8-pCPT-2′-O-Me-cAMP and its Sp-modified and hydrolysis-resistant form were comparable, thus formation of bioactive metabolic products [29] do not account for the platelet inhibitory effect. Interestingly, we also found that 8-pCPT-2′-O-Me-cAMP inhibited P-selectin expression elicited by the intracellular PKC agonist PMA (Fig. 2B), which initiates secretion of granules from platelets [30]. Upon stimulation by a strong agonist a sequence of platelet responses are initiated [11], including synthesis (e.g., TxA_2 and PAF) and secretion of secondary agonists like ADP from platelets granules. The secreted ADP elicit positive feedback through G_{i2}-coupled $P2Y_{12}$ receptors and involves activation of intracellular signalling molecules like PI3 K [12,15,31] and Rap1 [32], which increases the power of the primary agonist. In platelets Rap1B can be activated via CalDAG-GEFI-dependent and independent pathways [32,33], the latter requires activation of PKC, PKC-induced platelet secretion of ADP and subsequent $P2Y_{12}$ activation [34,35].

We studied the role of ADP-receptors in 8-pCPT-2′-O-Me-cAMP mediated inhibition, and found that in the presence of the extracellular ADP removing CP/CPK system, even high concentrations of 8-pCPT-2′-O-Me-cAMP failed to inhibit the PMA-provoked P-selectin expression in platelets (Fig. 2B). The activation by the PKC agonist is dependent on secreted ADP, and the fact that 8-pCPT-2′-O-Me-cAMP failed to inhibit activation when secreted ADP was removed suggests that this cAMP analogue functions as an ADP-receptor antagonist.

The Epac activator 8-pCPT-2′-O-Me-cAMP *per se* failed to activate Rap 1 (Rap 1-GTP), but contradictory blocked the strong thrombin- and TRAP-induced Rap 1 activation in platelets (Fig. 2C), as previously described by others [8]. The PKA antagonist Rp-cAMPS and Rp-8-Br-cAMPS did not counteract the inhibitory effect of 8-pCPT-2′-O-Me-cAMP on Rap 1 activation (Fig. 2C), suggesting that PKA is not involved. Interestingly, Rap1B can be activated by a CalDAG-GEFI-independent pathway [32,33], dependent on both PKC, secreted autocrine ADP and subsequent $P2Y_{12}$ receptor activation [34,35].

3.3. VASP Ser-157 and VASP Ser-239 phosphorylation are not affected by 8-pCPT-conjugated Epac analogues per se

It has been shown that several of the PDEs are inhibited by 8-pCPT-2′-O-Me-cAMP and Sp-8-pCPT-2′-O-Me-cAMP [1], enhancing SNP/cGMP and forskolin/cAMP-induced phosphorylation at VASP-239 (preferentially phosphorylated by PKG [36]) or VASP-157 (preferentially phosphorylated by PKA [36]), respectively.

To determine whether the 8-pCPT-conjugated Epac analogues by themselves can induce site-specific phosphorylation of VASP, we incubated human platelets for up to 15 min with 8-pCPT-2′-O-Me-cAMP or Sp-8-pCPT-2′-O-Me-cAMP, using the PKA-activator Sp-5,6-DCL-cBIMPS and the PKG activator 8-Br-PET-cGMP as positive controls. 8-Br-PET-cGMP strongly inhibits PDE2 [1,22], leading to increased cAMP, and can therefore be used as a positive control for site-specific phosphorylation at both VASP-239 and VASP-157. As shown in Fig. 3A and B, platelets incubated with either Epac-activator had no change in VASP phosphorylation after 15 min incubation. In this study we also monitored the effect on P-selectin expression after 5 min preincubation with the Epac analogues (Figs. 1 and 2). Thus, the inhibitory effect of 8-pCPT-conjugated Epac analogues on platelet activation cannot be explained by PDE inhibition, elevated cAMP and subsequent PKA stimulation.

3.4. 8-pCPT-2′-O-Me-cAMP docks similar to a $P2Y_{12}$ antagonist, but different from an agonist

We have thus identified a potential off target effect of 8-pCPT-2′-O-Me-cAMP on the purinergic platelet receptor $P2Y_{12}$. This receptor has received attention since it is a target for anti-thrombotic drugs and reliable models of the molecule docked with agonists and antagonists have been generated [24,37,38]. We docked 8-pCPT-2′-O-Me-cAMP into the binding site of $P2Y_{12}$, and compared with similar docking of the agonist 2MeSADP and the antagonist 2MeSAMP (Fig. 4). We found that whereas the phosphate groups of all three ligands to some degree formed H-bonds to R256, postulated to be important for activation [39], the adenine rings of 8-pCPT-2′-O-Me-cAMP and 2MeSAMP formed interactions with F177 (Fig. 4 B and D) rather than with Y259, which forms π–π interactions with the agonist 2MeSADP (Fig. 4F). Y259, together with R256 and K280 is shown to be important for activation of $P2Y_{12}$ [39].

The possibility is that the thiol-moiety in 8-pCPT-2′-O-Me-cAMP reacts with C97 or C175, which are targets for thiol-containing

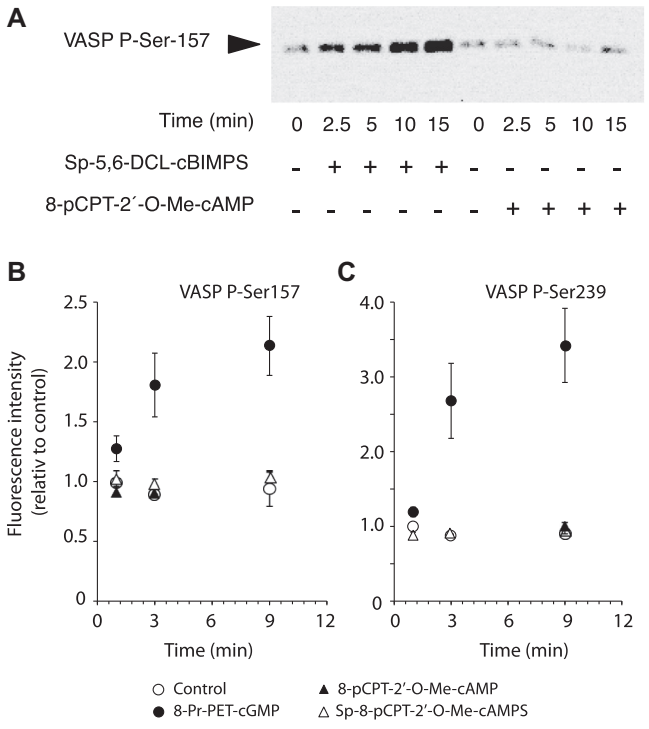

Fig. 3. 8-pCPT-2′-O-Me-cAMP does not provoke VASP phosphorylation in platelets. (A) Isolated platelets were treated with the PKA activator Sp-5,6-DCL-cBIMPS (0.5 mM) or 8-pCPT-2′-O-Me-cAMP (0.5 mM) for the indicated periods of time, and phosphorylation of VASP at Ser157 was visualized by western blotting as described in the Methods section. (B) and (C) Platelets were treated with 0.2 mM 8-Br-PET-cGMP to activate PKG and inhibit PDE's with subsequent elevation of cAMP levels, or the same concentrations of the Epac agonists 8-pCPT-2′-O-Me-cAMP or Sp-8-pCPT-2′-O-Me-cAMP, and phosphorylation of VASP at Ser157 (B) or Ser239 was assessed by flow cytometry as described in the Methods section. The data in B and C are average of 3–4 experiments and SEM.

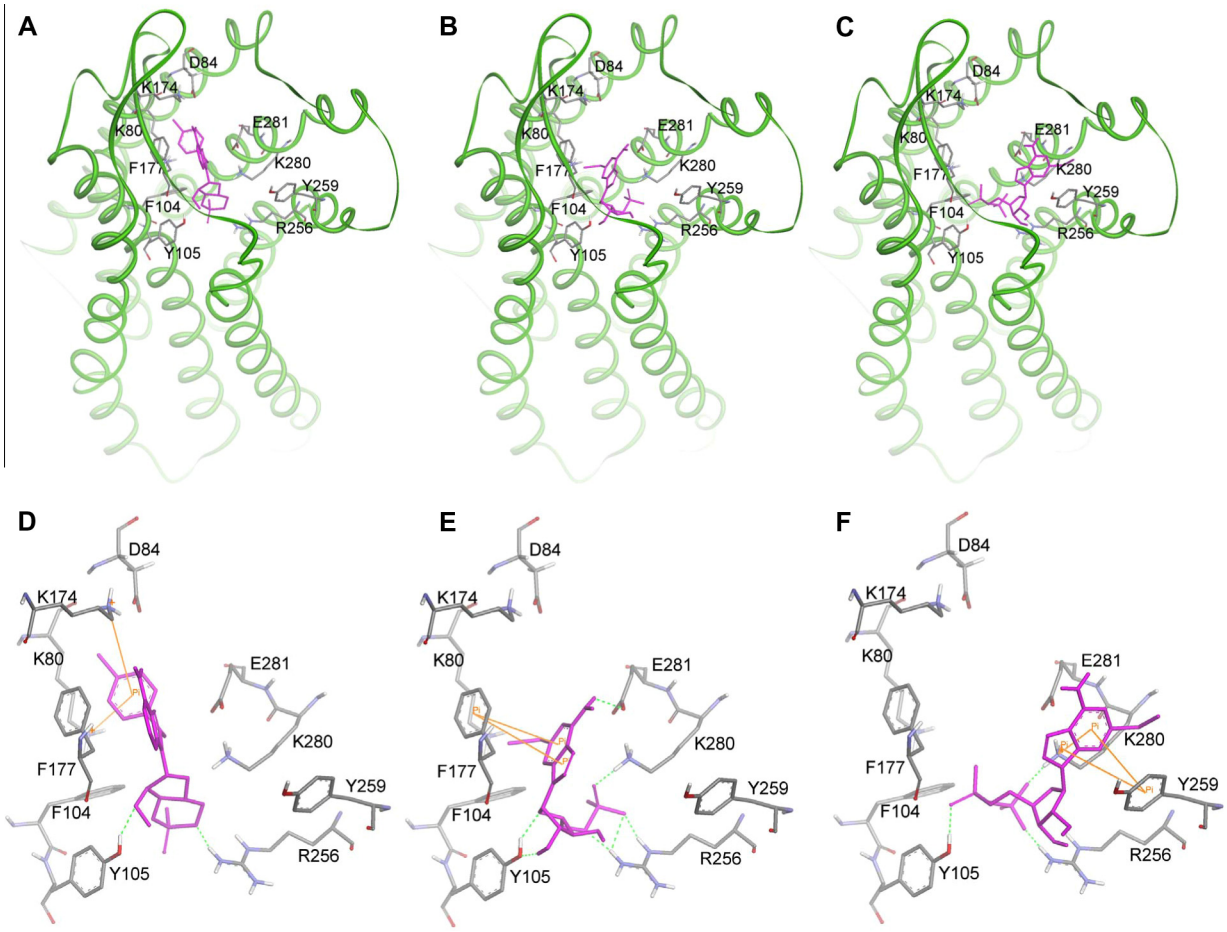

Fig. 4. *In silico* modelling of the binding of antagonists and agonists to $P2Y_{12}$. 8-pCPT-2′-O-Me-cAMP. (A,B), 2-Me-SAMP (antagonist, C, D), and 2-Me-SADP (agonist, E, F). The ligands are in pink, protein backbone shown in green in A, C, D, and close residues shown in grey. In B, D, E, only close residues and the ligand is shown. H-bonds are green dotted lines, and Pi-interactions orange lines. Settings and conditions for ligand preparation and docking into a $P2Y_{12}$R-CXC model [24] are described in the Methods section. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

antagonists [40] is unlikely for the short incubation periods in serum-free conditions used the present study.

Although the data presented in Fig. 4 does not allow for extensive discussion concerning details on interactions between residues in the ADP-binding core of $P2Y_{12}$ and 8-pCPT-2′-O-Me-cAMP, it appears that the two antagonists (Fig. 4A–D) interact differently from the agonist (Fig. 4E and F). In our system, the Epac-agonist 8-Br-2′-O-Me-cAMP docked differently from both 8-pCPT-2′-O-Me-cAMP, 2MeSAMP and 2MeSADP (not shown). 8-Br-2′-O-Me-cAMP was inactive at concentrations where we obtained full inhibition with 8-pCPT-2′-O-Me-cAMP (Fig. 2A and data not shown).

The primary target of the cAMP analogue 8-pCPT-2′-O-Me-cAMP is Epac [3,41]. Here we demonstrate an off-target effect of 8-pCPT-2′-O-Me-cAMP on the G-protein-coupled receptor $P2Y_{12}$ in platelets. We show that 8-pCPT-2′-O-Me-cAMP inhibits agonist-induced-GPCR-stimulated P-selectin expression in Epac1 deficient platelets, and that the 8-pCPT-substituated cAMP analogues appear to act as a $P2Y_{12}$ receptor antagonist. The importance of synergistic positive feedback loop by secreted ADP and TxA_2 are well documented for TRAP- and thrombin-induced platelet signalling [12–15,17,25], and ADP signalling via G_{i2}-coupled $P2Y_{12}$ receptors is essential for TxA_2-induced platelet activation via G_q/G_{13} coupled receptors, and vice versa [9,17,42]. Our data are consistent with such a scenario, thus suggesting that the 8-pCPT-2′-O-Me-cAMP analogue can antagonise both $P2Y_{12}$ and TxA_2 [8] receptors, whereas 8-Br-2′-O-Me-cAMP appears to interact less with the platelet activation machinery.

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