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Biochemical Pharmacology 64 (2002) 433–439

Biochemical Pharmacology

Inhibition of platelet P2Y12 and α2A receptor signaling by cGMP-dependent protein kinase

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Received 20 February 2002; accepted 8 May 2002

Abstract

The important role of cGMP and cGMP-dependent protein kinase (cGPK) for the inhibition of platelet activation and aggregation is well established and due to the inhibition of fundamental platelet responses such as agonist-stimulated calcium increase, exposure of adhesion receptors and actin polymerization. The diversity of cGMP binding proteins and their synergistic interaction with cAMP signaling in inhibiting platelets indicates that a variety of cGMP targets contribute to its antiplatelet action. Since stimulation of G_i-proteins was recently shown to be essential for complete platelet activation/aggregation, the possibility that G_i-signaling events are cGMP/cGPK targets was investigated. Thus, the effect of elevated cGMP levels and selective cGPK activation on purinergic and adrenergic receptor-evoked decrease of platelet cAMP content was closely examined. Experiments with a selective activator of cGPK demonstrate for the first time a cGMP-caused G_i-protein inhibition and our data suggest that this effect is mediated by cGPK. Considering the essential role of G_i-signaling for platelet activation, we propose that inhibition of G_i-mediated signaling by cGMP/cGPK is an important mechanism of action underlying the platelet inhibition by cGMP-elevating endothelium derived factors and drugs.

Keywords: Adenylyl cyclase; Purinergic signaling; Adrenergic signaling; Phosphodiesterase; cGMP-dependent protein kinase; Endothelial factors

1. Introduction

In vivo, platelets are continually exposed to the endothelial-derived factors nitric oxide (NO) and prostacyclin (PG-I_2) which inhibit and limit unwarranted platelet activation [1,2]. NO and PG-I_2 stimulate the formation of cGMP and cAMP, respectively, by direct activation of platelet soluble guanylyl cyclase (GC) and a G_s-protein coupled prostanoid receptor on platelet membranes [2]. Elevated cyclic nucleotide levels activate the corresponding cyclic nucleotide-dependent protein kinase and phosphorylation of their substrates [3]. In platelets, this contributes to the inhibition of stimulated intracellular calcium signaling [4,5], fibrinogen binding [6], adhesion [7], and aggregation of human platelets [1]. The cyclic nucleotides regulate, but are also regulated by, phosphodiesterases (PDE) [8]. In human platelets, three different PDE subtypes were identified [9,10]: the cGMP-stimulated unspecific phosphodiesterase type II (PDE 2), the cGMP-inhibited and cAMP specific phosphodiesterase type III (PDE 3) and the cGMP stimulated and cGMP specific phosphodiesterase type V (PDE 5). Synergistic inhibitory action of cyclic nucleotides on platelet function has been frequently described [11,12]. This phenomenon can be, at least partially, attributed to the inhibition of PDE 3 by cGMP [13,14]. If this PDE is inhibited, cAMP decomposition is reduced leading to an increase of cAMP content. On the other hand, cAMP accumulation is limited by PDE 2 which actually is stimulated by cGMP [15].

Activation of human platelets is mediated primarily by G-protein coupled receptors [16]. For irreversible platelet aggregation, concomitant stimulation of G_q- and G_i-protein is essential. Platelet stimulation by adrenalin is mediated by an α-adrenoceptor of the α2A subtype [17] linked to G_i-protein. In mice platelets the α2A receptor has been shown to couple to G_z, a protein of the G_i-family [18].

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Abbreviations: NO, nitric oxide; PG-I_2, prostacyclin (prostaglandin I_2); GC, guanylyl cyclase; AC, adenylyl cyclase; PDE, phosphodiesterase; IP3, 1,4,5-inositol trisphosphate; cGPK, cyclic GMP dependent protein kinase; 8CPT-cGMP, 8-(*p*-chlorophenylthio) cyclic-guanosin 5′-monophosphate; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; SNP, sodium nitroprusside; PG-E1, prostaglandin E_1; VASP, vasodilator stimulated phosphoprotein.

0006-2952/02/$ – see front matter © 2002 Published by Elsevier Science Inc.
PII: S0006-2952(02)01113-9

However, this has not been confirmed for human platelets yet. Thus, adrenalin cannot induce complete platelet aggregation but amplifies platelet responses to stimulation by other partial agonists coupling to G_q-protein [16].

ADP stimulated aggregation of human platelets is mediated by different purinergic receptors [16,19,20], of which two are coupled to G-protein mediated signaling pathways: the P2Y1 receptor causing calcium mobilization *via* G_q [21,22] and the recently cloned P2Y12 receptor [23–26] mediating inhibition of adenylyl cyclase (AC) by stimulation of a G_i-protein [22]. Recent studies on the mechanism of action of ADP-receptor mediated activation of platelet aggregation revealed that ADP stimulated activation of G_i-protein leading to reduced cAMP levels and decreased phosphorylation of the vasodilator stimulated phosphoprotein (VASP) are key events for platelet activation [27].

Experiments with specific inhibitors of P2Y1 and P2Y12 receptors [19,20], human platelets deficient for these receptors [28,29] and P2Y1 or P2Y12 knock-out mice [30–32], proved that both pathways are necessary for full platelet aggregation.

On the basis of the pathways mentioned regulating platelet cyclic nucleotides *via* G_i-protein and PDEs, we hypothesize a diverse crosstalk of cAMP and cGMP mediated mechanisms. Here we show that elevated cGMP levels and selective stimulation of cGMP-dependent protein kinase (cGPK) cause an inhibition of G_i-protein mediated pathways which may be an important component of the antiplatelet effects of cGMP elevating agents.

2. Materials and methods

8CPT-cGMP and EHNA were obtained from Biolog. All other chemicals were obtained from Sigma.

2.1. Platelet preparation

Platelets were prepared from freshly drawn whole human blood according to the published protocol [19]. The blood was obtained by venipuncture from healthy volunteers who had not received any medical treatment within the last 2 weeks. The blood was collected in a citrate buffer (100 mM sodium citrate, 7 mM citric acid, 140 mM glucose, pH 6.5) without addition of any anticoagulant or antiaggregant. Platelet rich plasma was separated by centrifugation for 20 min at 300 *g* (Sigma 3K-1). The platelet rich plasma was then removed with a plastic pipette and transferred into plastic tubes for the experiments.

2.2. Determination of platelet cAMP content

For the experiments 0.3 mL aliquots of platelet suspension in siliconized Eppendorf caps were either incubated solely with ethanol (1% ethanol (v/v)) for 2 min (base control), 1 μM prostaglandin E_1 (ethanol solved, final concentration 1% ethanol (v/v)) as stimulator of AC, 5 μM ADP, 5 μM adrenalin, 100 μM 2′,5′-dideoxyadenosine or combinations of the above for 2 min. GC stimulation was accomplished by preincubation of PRP for 2 min with 0.1 mM sodium nitroprusside (SNP). Stimulation of cGMP dependent protein kinase was achieved by 20 min preincubation with 1 mM 8-(*p*-chlorophenylthio) cyclic-guanosin 5′-monophosphate (8CPT-cGMP) before addition of ADP and PG-E_1. The incubation was stopped with 0.5 mL 70% (v/v) ice cold ethanol and kept on ice for 30 min. The precipitate was pelleted by centrifugation for 10 min at 5,000 *g* at 4°. The supernatant was transferred to an Eppendorf cap and the precipitate was extracted twice with 0.5 mL 70% (v/v) ethanol. The extracts were combined and evaporated in membrane pump vacuum. The resulting extract was solved in 0.5 mL of assay buffer (50 mM sodium acetate, pH 5.8). The cAMP determination was performed using the Amersham Biotrak cAMP RIA kit.

3. Results

As expected, platelets treated with 1 μM PG-E_1 showed a more than 10-fold increase in cAMP content (80–100 pmol/10^9 platelets). This increase in cAMP is inhibited by ADP stimulation. With 5 μM ADP a reduction of the prostaglandin E_1 (PG-E_1) stimulated cAMP increase by 65% is observed (Fig. 1). SNP at a concentration of 0.1 mM does not affect the basal human platelet cAMP level. However, PG-E_1 evoked cAMP increase was remarkably reduced if the platelets were pretreated with 0.1 mM SNP, compared to the cells treated with PG-E_1 alone. The cAMP level in the SNP treated cells reached only about 60% of the PG-E_1 control. By combination of ADP stimulation and SNP treatment consequently an even more pronounced reduction of elevated cAMP content should be expected. In fact quite the contrary was observed. The cAMP level obtained by combined treatment with 0.1 mM SNP, 5 μM ADP and 1 μM PG-E_1 was 14% lower than in PG-E_1 and SNP treated platelets, but 17% higher than in cells treated with ADP and PG-E_1 (Fig. 1). A similar but more distinct effect was observed by stimulation of platelet α2A receptors (Fig. 2). Adrenalin stimulation led to a reduction of PG-E_1 evoked cAMP increase by 80%. SNP again had an inhibitory effect on stimulated reduction of platelet cAMP level. The cAMP level in platelets treated with 0.1 mM SNP, 1 μM PG-E_1 and 5 μM adrenalin was reduced by 70% compared to the maximal cAMP content obtained with 1 μM PG-E_1 alone.

The cGMP formed upon treatment with SNP does not only activate cGPK but also cGMP-stimulated PDE 2. To circumvent problems arising from these additional effects, the experiments were also carried out with the selective cGPK stimulant 8CPT-cGMP [33]. 8CPT-cGMP is

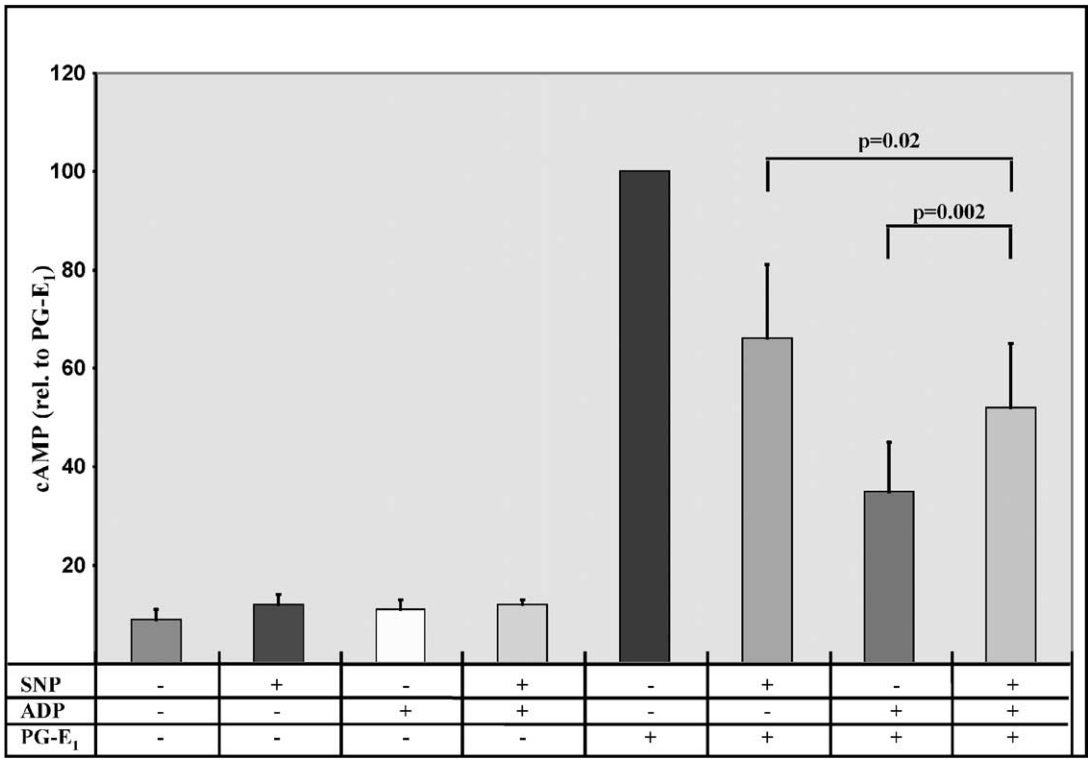

Fig. 1. ADP evoked inhibition of AC through activation of G_i-protein in human platelets is inhibited by stimulation of GC with SNP. Human platelets were treated either with 5 μM ADP, 1 μM PG-E_1, 0.1 mM SNP or combinations of these. The cAMP concentrations are relative to the amount of cAMP formed after stimulation with 1 μM PG-E_1. The data shown represent means of six independent experiments with different blood donors ± SD and, where indicated, *P*-values from ANOVA tests.

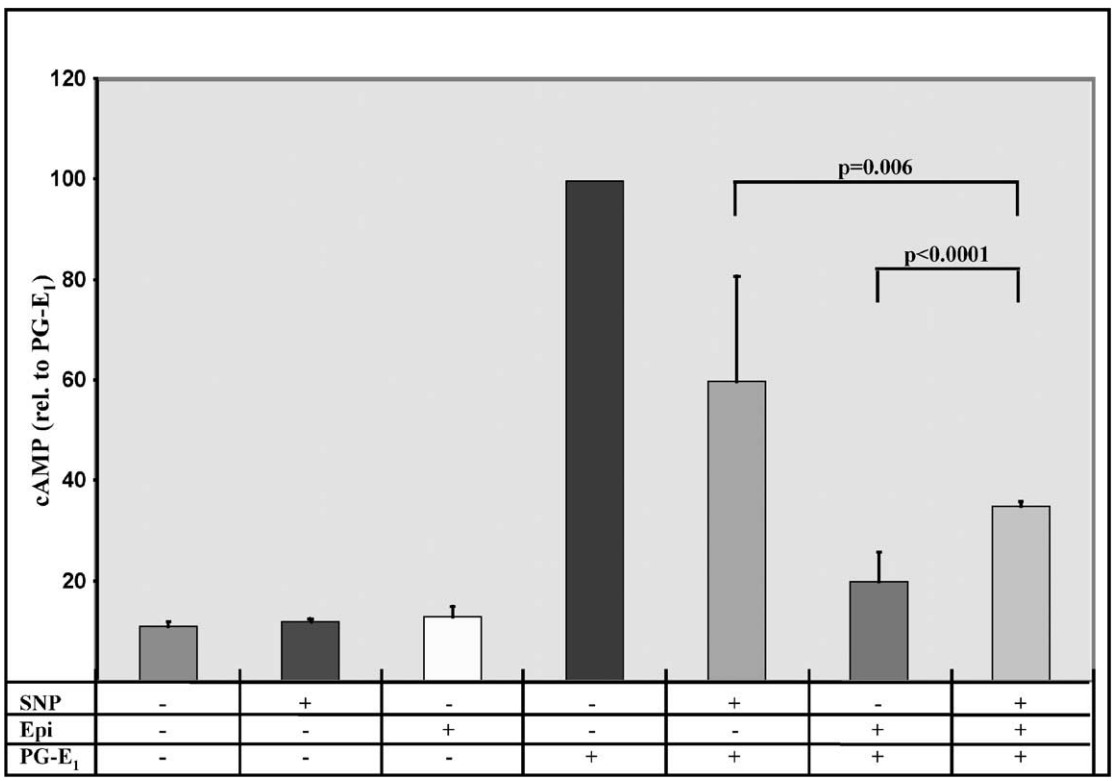

Fig. 2. Inhibition of AC caused by adrenalin in human platelets is inhibited by stimulation of GC with SNP. Human platelets were treated either with 5 μM adrenalin (Epi), 1 μM PG-E_1, 0.1 mM SNP and combinations of these. The cAMP concentrations are relative to the amount of cAMP formed after stimulation with 1 μM PG-E_1. The data shown represent means of four independent experiments with different blood donors ± SD and, where indicated, *P*-values from ANOVA tests.

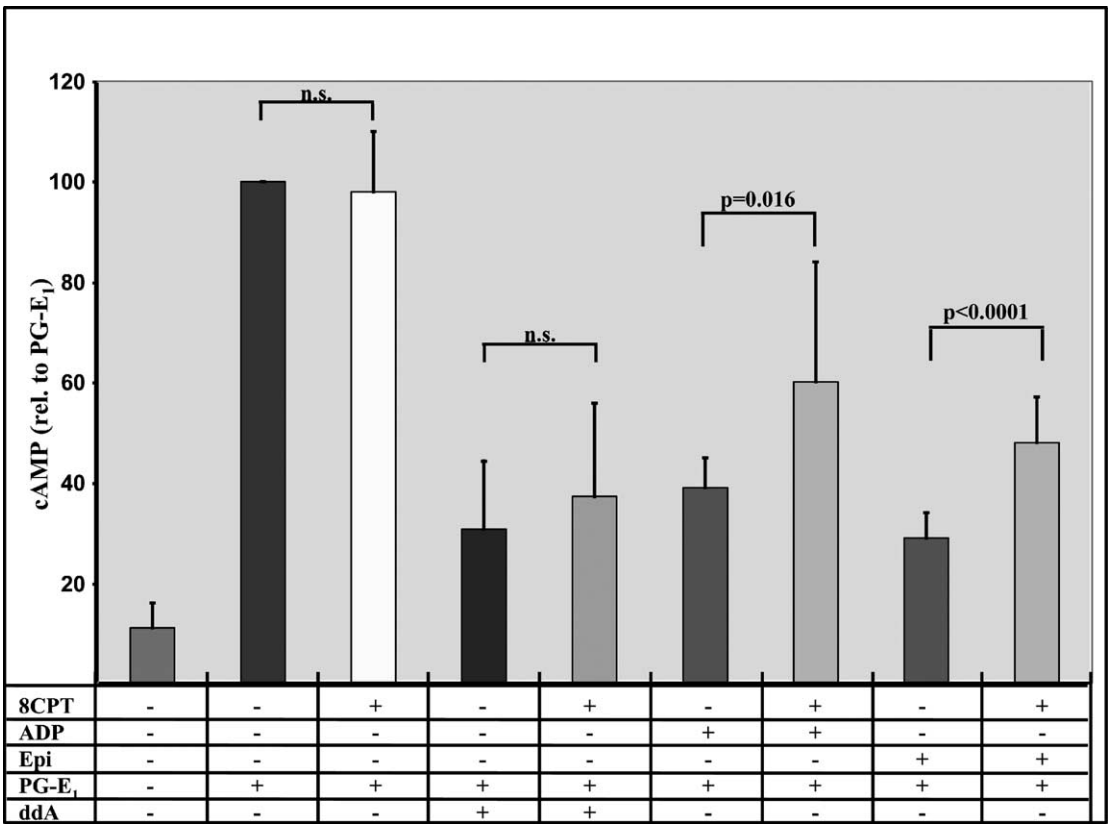

Fig. 3. Effect of the specific cGPK activator 8CPT-cGMP on cAMP accumulation and stimulation of G_i-protein by ADP or adrenalin. Human platelets were treated either with 5 μM adrenalin (Epi), 5 μM ADP, 1 μM PG-E_1, 100 μM 2′,5′-dideoxyadenosine, 1 mM 8CPT-cGMP (8CPT) and combinations of these. The cAMP concentrations are relative to the amount of cAMP formed after stimulation with 1 μM PG-E_1. The data shown represent means of five independent experiments with different blood donors ± SD and, where indicated, *P*-values from ANOVA tests.

a lipophilic compound and can easily permeate platelet membranes, activates cGPK without affecting PDEs and is stable with regard to hydrolysis by PDEs [33]. The basal human platelet cAMP content remained unchanged after preincubation with 1 mM of the cGMP analogue (Fig. 3). In contrast to SNP treatment 1 mM 8CPT-cGMP did not influence PG-E_1 evoked cAMP increase. Furthermore, 8CPT-cGMP did not alter the inhibitory effect of AC inhibitor 2′,5′-dideoxyadenosine on PG-E_1 stimulated cAMP increase, which indicates that the prostaglandin stimulated pathway is not affected by cGPK. The inhibition of PG-E_1 stimulated increase of platelet cAMP is therefore presumably caused by stimulation of the cGMP stimulated PDE 2. The ADP or adrenalin stimulated reduction of PG-E_1 caused cAMP increase however was—as observed with SNP pretreated platelets—markedly reduced by cGPK activation (Fig. 3).

To exclude potential side effects of 8CPT-cGMP on PDE 2 the PDE 2 inhibitor EHNA was used in the following experiments. EHNA had no significant effect on PG-E_1 stimulated cAMP increase in platelets at 20 μM, the concentration which has already been used in experiments with human platelets [34]. Under these conditions inhibition of cAMP accumulation by ADP was not influenced by EHNA pretreatment (data not shown). Inhibition of ADP stimulation by activation of cGPK with 8CPT-cGMP remained unaffected by EHNA as well. This clearly proves that PDE 2 is not involved in the inhibition of AC by ADP stimulation and is not stimulated by 8CPT-cGMP either.

4. Discussion

It is well established that a fine balance of platelet activation and platelet inhibition is essential in primary hemostasis. Recent investigations on platelet biochemistry have shown that platelet aggregation is the result of synergistic activation of G_q-protein mediated Ca^{2+}-mobilization and G_i-protein mediated inhibition of AC [16,19,20]. Inhibition or lack of one of these pathways is sufficient to prevent platelet aggregation. This could be shown with specific inhibitors of P2Y1 and P2Y12 receptors [19,20], with human platelets deficient for these receptors [28,29] as well as in P2Y1 and P2Y12 deficient mice [30–32] and mice lacking the α-subunit of the G_q- [35] or G_i-protein [36]. On the other hand, platelet aggregation can be achieved with stimulants exclusively activating G_q and G_i, if given in combination [16].

Cyclic nucleotide elevating substances are potent inhibitors of platelet aggregation. *In vivo*, platelet inhibition by

the endogenous cyclic nucleotide elevating agents NO and PG-I_2 is supposedly one of the major mechanisms maintaining blood flow [11]. Exogenous stimulants of nucleotide cyclases like NO donors or prostaglandins are acting anti-aggregatory as well [37] and have proved efficient in inhibiting platelet aggregation. Activation of guanylyl and AC have a synergic effect in inhibiting platelet aggregation. The mechanisms by which these substances exert their inhibitory action on platelet aggregation have been extensively studied but are yet not completely understood. It could be shown that stimulants of guanylyl and AC like endothelial factors as well as activators of cGPK are involved in the inhibition of platelet calcium responses [5], fibrinogen binding [6] and aggregation [11]. Furthermore, the inhibitory role of the cGPK for platelet function *in vitro* and *in vivo* has been validated in cGPK-deficient mice [38]. Nevertheless, the molecular details underlying this inhibitory function of cGPK have not been identified yet and appear to involve more than one target and mechanism. Clearly, activators of soluble GC and cGPK inhibit agonist-induced, G_q-protein mediated platelet calcium responses [1,5] and impair agonist induced fibrinogen binding and integrin $\alpha_{IIb}\beta_{III}$ activation [6]. In the latter case, this involves VASP and VASP phosphorylation as has been confirmed by the analysis of platelets from VASP-deficient mice [39,40]. Yet another mechanism of cGMP function in platelets (which is cGPK-independent) is the inhibition of PDE 3 and subsequent potentiation of cAMP signaling [10,34].

Under certain conditions, cGMP may also reduce cAMP by stimulating PDE 2. In agreement with previous studies, basal cAMP levels were not significantly affected by GC/cGMP stimulating agents whereas cAMP increase upon stimulation of AC by PG-E_1 was reduced by about 40%. In agreement with published data [34] the lack of any effect of the cGPK activator 8CPT-cGMP (described later) on PG-E_1 stimulated cAMP increase in platelets indicates that the observed reduction in cAMP increase in SNP treated cells most likely results from cGMP stimulation of PDE 2.

In the present study, we provide evidence for another, new mode of action for cGPK-mediated inhibition of platelet activation. Our present data show that SNP impairs the ADP caused reduction of PG-E_1 stimulated platelet cAMP content. For example, the PG-E_1 stimulated cAMP response in SNP-pretreated platelet was reduced by ADP only to an extent of about 45% rather than 35% in the absence of SNP (Fig. 1). This indicates an additional mechanism which antagonises P2Y12 receptor mediated inhibition of AC. The effect is not limited to the purinergic response, but is also observed in adrenergic stimulation of G_i-protein (Fig. 2). The data obtained with SNP do not clarify whether the observed effect on G_i-stimulation is connected to PDE 2 and/or cGPK activation through cGMP. Since an cGPK effect was suspected, the effect of a selective, well established cGPK activator, 8CPT-cGMP, was investigated. Under the conditions used, 8CPT-cGMP neither affects the allosteric nor the catalytic properties of platelet PDE, therefore allowing differentiation of cGMP-dependent pathways [33]. SNP-induced inhibition of the purinergic and adrenergic G_i-stimulation was reproduced by 8CPT-cGMP indicating the participation of cGPK in this regulatory mechanism. In fact, impairment of the inhibition of AC by ADP and adrenalin was reduced by 8CPT-cGMP to about the same extent as SNP while the PG-E_1 stimulated cAMP increase remained unaffected by 8CPT-cGMP. These results indicate that the inhibition of ADP and adrenalin caused G_i-stimulation is mediated by cGPK.

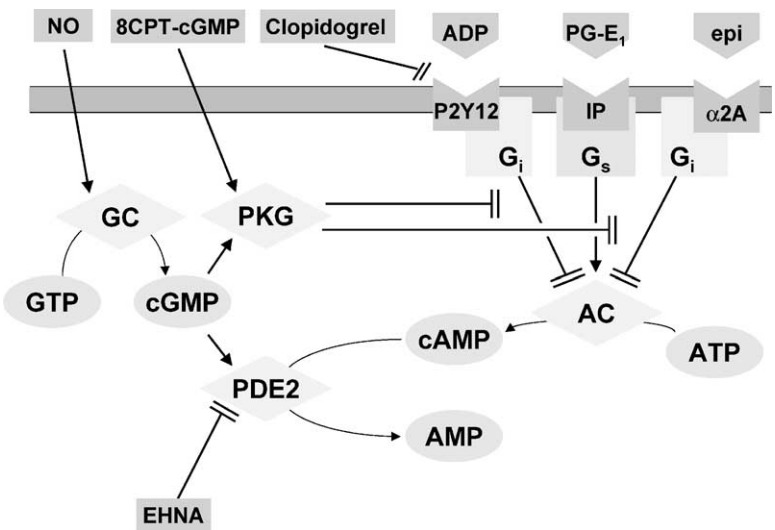

Fig. 4. Crosstalk of platelet cyclic nucleotide dependent signaling pathways. PG-E_1 stimulates formation of cAMP by activation of G_s-protein and activation of AC. Stimulation of GC by NO donors limits cAMP accumulation by stimulation of the cAMP degrading PDE 2. ADP, adrenalin and thrombin cause activation of G_i-protein. The antiplatelet drug clopidogrel is an inhibitor of the P2Y12 ADP receptor. cGPK is stimulated by cGMP or the cGMP analogue 8CPT-cGMP.

However, the precise biochemical target(s) of cGPK in this signaling cascade remains to be identified since our present data do not clarify whether platelet signal transduction is intercepted at the level of the receptor, receptor-G-protein coupling or G-protein effector coupling or function which is not only true for G_i-protein coupling as studied here but also for previously studied G_q-protein coupling. *In vitro*, the thromboxane receptor [41], the IP3 receptor [42], an IP3 receptor associated PKG substrate [43], and phospholipase C β3 [44] were shown to be cGPK substrates, but the functional *in vivo* relevance of these observations remains to be established. Possibly, a common target in platelet signal transduction is affected by cGPK as it is observed in stimulated increase of platelet calcium levels which is inhibited by cGPK activation regardless of the stimulant [4,5].

In conclusion, activation of cGPK by NO donors or cGMP analogues inhibited purinergic and adrenergic G_i-protein stimulation thereby diminishing purinoceptor and adrenoceptor mediated AC inhibition (Fig. 4). Since small effects on platelet cAMP levels are known to have major functional effects [45] and since purinergic/G_i-signaling is essential for platelet activation, this new mechanism of cGMP/cGPK could significantly contribute to the NO/cGMP-caused inhibition of platelet activation and aggregation *in vivo*. Our data may therefore contribute to the understanding of the synergistic action of cyclic nucleotide elevating agents and explain the potent antiaggregatory effect of the endothelial factors *in vivo*. It is now well established that an endothelial dysfunction is an important component of a variety of vascular diseases. One consequence of impaired endothelial function and its associated diminished NO/cGMP signaling could be an enhanced signaling of the G_i-coupled receptors P2Y12 and α2A. This leads us to the conclusion that therapeutic inhibition of P2Y12 signaling by clopidogrel may at least partially substitute a normal endothelial response otherwise impaired or even lost due to disease.

Acknowledgments

This study was supported by a project grant form Sanofi-Synthelabo and Brystol Myers Squibb.

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