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Control of platelet activation by cyclic AMP turnover and cyclic nucleotide phosphodiesterase type-3

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Abstract

Prostaglandin-induced cAMP elevation restrains key signaling pathways in platelet activation including Ca^{2+} mobilization and integrin αIIbβ3 affinity regulation. We investigated how cAMP turnover by cyclic nucleotide phosphodiesterases (PDEs) regulates platelet activation. In washed human platelets, inhibition of all PDEs and also specific inhibition of PDE3 but not of PDE5 suppressed thrombin-induced Ca^{2+} responses. The effect of general PDE or PDE3 inhibition was accompanied by an increase in cAMP, and potentiated by Gs stimulation with prostaglandin E_1. In platelet-rich plasma, general or PDE3 inhibition blocked platelet aggregation, integrin activation, secretion and thrombin generation. In contrast, inhibition of PDE5 increased the cGMP level, but without significant influence on aggregation, αIIbβ3 activation, secretion or procoagulant activity. Nitroprusside (nitric oxide) potentiated the effect of PDE5 inhibition in elevating cGMP. Nitroprusside inhibited platelet responses, but this was accompanied by elevation of cAMP. Together, these results indicate that cAMP is persistently formed in platelets, independently of agonist-induced Gs stimulation. PDE3 thus functions to keep cAMP at a low equilibrium level and reduce the cAMP-regulated threshold for platelet activation. This crucial role of PDE3, but not of PDE5, extends to all major processes in thrombus formation: assembly of platelets into aggregates, secretion of autocrine products, and procoagulant activity.

Keywords: Aggregation; Calcium; Cyclic-AMP; Cyclic-GMP; Phosphodiesterase; Platelets

1. Introduction

During thrombus formation, platelets form vaso-occlusive aggregates and expose coagulation-active phospholipids at their surface. Key platelet agonists such as thrombin, ADP and thromboxane stimulate Gq-protein coupled receptors, causing phospholipase C activation and a subsequent rise in cytosolic $[Ca^{2+}]_i$ [1,2]. Elevated $[Ca^{2+}]_i$ mediates filopod formation, secretion of autocoids, and also exposure of procoagulant phosphatidylserine (PS) at the platelet surface [3,4]. Simultaneous activation of other G proteins, including Gi, Gz and G12/13, results in affinity regulation of the fibrinogen-binding integrin αIIbβ3 and platelet assembly into aggregates [5,6]. Both Ca^{2+} signaling and integrin activation are potently inhibited by endothelial-derived prostacyclin, which stimulates adenylate cyclase via the Gs protein, resulting in activation of cAMP-dependent protein kinase A [7,8]. The latter enzyme phosphorylates and down-regulates key proteins in Ca^{2+} mobilization and αIIbβ3 integrin activation [9,10].

Previously, we have reported that the 'basal' level of cAMP in resting platelets restricts various key activation processes such as Ca^{2+} mobilization, secretion and integrin-dependent aggregation. We demonstrated that epinephrine, and other agonists that signal via Gi/z, enhance these platelet responses by lowering the cAMP concentration [11]. This implicates that platelets—even in the absence of Gs-stimulating prostaglandins—exhibit ongoing turnover of cAMP formation and breakdown, to reach an equilibrium cAMP level which is still sufficiently

Abbreviations: AMC, 7-amido-4-methyl coumarin; $[Ca^{2+}]_i$, cytosolic-free calcium concentration; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; IBMX, 3-isobutyl-1-methyl xanthine; PDE, phosphodiesterase; PRP, platelet-rich plasma; PGE_1, prostaglandin E_1; SNP, sodium nitroprusside.

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high to limit platelet activation. This implies that cyclic nucleotide-dependent phosphodiesterases (PDEs), controlling cAMP hydrolysis, have a regulatory effect on platelet activation even in the absence of Gs-stimulating agonists.

Platelets contain at least three types of PDEs. These are cGMP-stimulated PDE2 and a cGMP-inhibited PDE3*A*, both of which use cAMP and cGMP as substrates; and cGMP-specific PDE5, which hydrolyzes only cGMP [12,13]. There is significant cross-talk between the cAMP and cGMP signaling pathways, as elevated cGMP can exert an inhibitory effect on PDE3*A*, which results in a net increase in cAMP level [14]. In addition, high cGMP stimulates PDE2 and can thus decrease cAMP [15]. This cross-talk has recently received new attention with the finding that the guanylate cyclase/cGMP/protein kinase G pathway might activate rather than inhibit platelets under certain conditions [16]. This would suggest that the role of cGMP-specific PDE5 in platelet activation is different from that suggested in earlier papers [12,14,17]. Precise knowledge of the function of PDE5 in platelets is of clinical importance, because of the current use of the PDE5 inhibitor dipyridamole in the secondary prevention of ischemic stroke [18,19].

In this paper we examined the possible function of PDEs to maintain steady-state levels of cAMP and cGMP even in the absence of agonist-evoked adenylate (prostaglandin) and guanylate cyclase (nitric oxide) stimulation. We describe that PDE3 lowers cAMP even in resting platelets, and thereby controls major platelet reactions in thrombus formation, such as secretion, aggregation and procoagulant activity. In contrast, PDE5 was active in lowering cGMP, but its inhibition did not significantly influence platelet reactions.

2. Materials and methods

2.1. Materials

Apyrase, dipyridamole, *erythro*-9-(2-hydroxy-3-nonyl)-adenine (EHNA), human α-thrombin, 3-isobutyl-1-methyl xanthine (IBMX), milrinone [1,6-dihydro-2-methyl 6-oxo-(3,4′-bipyridine)5-carbonitrile], zaprinast [1,4-dihydro-5-(2-propoxyphenyl)-7*H*-1,2,3-triazolo-(4,5-*d*)pyrimidine-7-one] and zardaverine [6-(4-difluoromethoxy-3-methoxyphenyl)-3(2*H*)-pyridazinone] were obtained from Sigma. PAR1-activating hexapeptide SFLLRN was from IHB. Fura-2 penta-acetoxymethyl ester was purchased from Molecular Probes; aspirin (lysine acetylsalicylate) was from Lorex Synthelabo; papaverine (6,7-dimethoxy-1-veratrylisoquinoline) and Ro20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] were from Alexis; sildenafil was from Pfizer; 2′,5′-dideoxyadenosine and SQ22536 were from Biomol; prostaglandin E_1 (PGE_1) and sodium nitroprusside (SNP) were from Janssen; D-Phe-Pro-Arg chloromethyl ketone (PPACK) and adenosine deaminase were from Calbiochem. Recombinant human tissue factor was from Dade, and fluorescent thrombin substrate *Z*-Gly-Gly-Arg 7-amido-4-methyl coumarine (*Z*-GGR-AMC) was from Bachem. Fluorescein isothiocyanate (FITC)-labeled antibody PAC-1 against activated integrin αIIbβ3 was obtained from Becton and Dickinson and FITC-labeled AK6 antibody against CD62P from CBL-Sanquin.

2.2. Platelet preparation and Ca^{2+} measurements

Blood was freshly drawn from healthy volunteers, who gave informed consent and had not taken medication for at least 2 weeks. For Fura-2 loading, blood was collected in 13.3 mM citrate, 8.7 mM citric acid and 30 mM glucose (f.c.). Platelet-rich plasma (PRP) was prepared by centrifugation, and incubated with aspirin (100 μM) and apyrase (0.1 U ADPase/ml). Loading with Fura-2 was performed as described [20]. After two wash steps, the platelets were resuspended in Hepes buffer pH 7.45, containing 136 mM NaCl, 10 mM glucose, 5 mM Hepes, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin and apyrase (0.1 U ADPase/ml).

Changes in cytosolic $[Ca^{2+}]_i$ were continuously measured in stirred suspensions of Fura-2-loaded platelets (1×10^8 ml^{-1}) in Hepes buffer with 1 mM $CaCl_2$ at 37 °C by ratio fluorometry [21]. Platelets were pre-incubated at 37 °C with indicated PDE inhibitor (dissolved in DMSO at 500-fold higher concentration). Where indicated, 5-min time integrals of agonist-induced rises in $[Ca^{2+}]_i$ were determined to measure amounts of Ca^{2+} signal generation [20]. Calibrations of $[Ca^{2+}]_i$ were corrected for fluorescence from PDE inhibitors. As shown before, platelet Ca^{2+} responses are parametrically distributed [21].

Blood was collected on citrate (12.9 mM trisodium citrate, f.c.) for measurements of platelets in plasma. PRP was obtained by centrifugation at $150 \times g$ for 15 min, and brought to a count of $1.5–2 \times 10^8$ platelets/ml with autologous platelet-poor plasma. Platelet-poor plasma was prepared by centrifuging the PRP at $1800 \times g$ for 10 min, and platelet-free plasma by another centrifugation step at $18{,}000 \times g$ for 10 min.

2.3. cAMP and cGMP measurements

Samples of 200 μl of platelets (0.4×10^8 cells) were withdrawn from incubations of washed platelets in Hepes buffer pH 7.45. The samples were added to ice-cold ethanol (70%, f.c.), and frozen in liquid nitrogen. After thawing and centrifugation ($350 \times g$, 15 min), the supernatants were dried under nitrogen and dissolved in 100 μl assay buffer used to measure cAMP or cGMP using Biotrak enzyme immunoassay systems (Amersham Pharmacia Biotech).

2.4. Platelet aggregation and flow cytometry

Platelet aggregation was measured of aspirin-treated PRP (2×10^8 platelets/ml), recalcified with 16.6 mM

$CaCl_2$ and 40 μM PPACK (37 °C), resulting in a final plasma free Ca^{2+} concentration of about 2 mM. The PRP was pre-incubated with PDE inhibitor as desired, and activated with 10 μM SFLLRN. Aggregation was recorded from changes in light transmission. For flow cytometric analysis, aspirin-treated PRP (2.0×10^8 platelets/ml) was pre-incubated with PDE inhibitor, activated with 10 μM SFLLRN for 5 min, and evaluated on secretion with FITC-labeled anti-CD62P, or on activated integrin αIIbβ3 with FITC-labeled PAC-1 antibody [11]. Analyses were performed on a Epics XL flow cytometer from Coulter Electronics.

2.5. Thrombin generation

Thrombin generation was measured in freshly isolated, citrated PRP, basically as described [22]. Briefly, PRP diluted with autologous PPP to 1.5×10^8 platelets/ml was pre-incubated with indicated PDE inhibitor for 5 min (37 °C). Samples of 80 μl were transferred to wells of a 96-wells plate (Imunolon 2HB, Dynex Technologies), already containing 20 μl of 3 pM recombinant human tissue factor. After 5-min incubation at 37 °C in the presence of PDE inhibitor or vehicle, coagulation was started by automated addition of 20 μl of Hepes buffer pH 7.45 containing 2.5 mM *Z*-GGR-AMC, 100 mM $CaCl_2$ and 6% (w/v) bovine serum albumin. After mixing for 10 s, thrombin formation was followed in time by measuring the fluorescence of free AMC every 20 s using a Fluoroskan Ascent 374 well-plate reader (Labsystems). Samples were measured in quadruplicate; controls were run with autologous platelet-free plasma to check for microvesicle-dependent thrombin generation, which interferes with the platelet-dependent thrombin formation. Purified α-thrombin was used to convert AMC fluorescence values to thrombin concentrations. First-derivative traces, corrected for contribution of α_2-macroglobulin-bound thrombin, gave curves of thrombin formation and inactivation [23].

2.6. Statistics

Raw data were analyzed by ANOVA with Bonferroni post hoc analysis, and significance was determined at $P < 0.05$.

3. Results

3.1. Suppression of agonist-induced Ca^{2+} mobilization and integrin αIIbβ3 activation by inhibition of PDE3 but not of PDE5

Previously, we have demonstrated that the 'basal' level of cAMP in resting platelets has a small but significant suppressive effect on Ca^{2+} mobilization, secretion and αIIbβ3-dependent aggregation [11]. This suggests that platelets have appreciable adenylate cyclase activity and cAMP production even in the absence of added Gs-stimulating receptor agonist. To investigate this, we compared the effects of PDE inhibition on platelet Ca^{2+} signaling in the presence and absence of a low dose of Gs-stimulating PGE_1. In Fura-2-loaded platelets, we measured Ca^{2+} responses induced by the Gq- and Gi-stimulating agonist, thrombin, applied at sub-maximal concentration. Platelets were treated with aspirin and apyrase to prevent secondary reactions due to autocrine thromboxane A_2 and ADP, respectively. As shown in Fig. 1A, in the presence of a specific PDE3 inhibitor like milrinone [24], even at a low dose of 1 μM, thrombin-induced increases in $[Ca^{2+}]_i$ were substantially suppressed. In contrast, PDE5 inhibition with dipyridamole [25], even at higher concentrations (20 μM), did not influence this response. Compared to the maximal $[Ca^{2+}]_i$ rise under control conditions of 623 ± 112 nM,

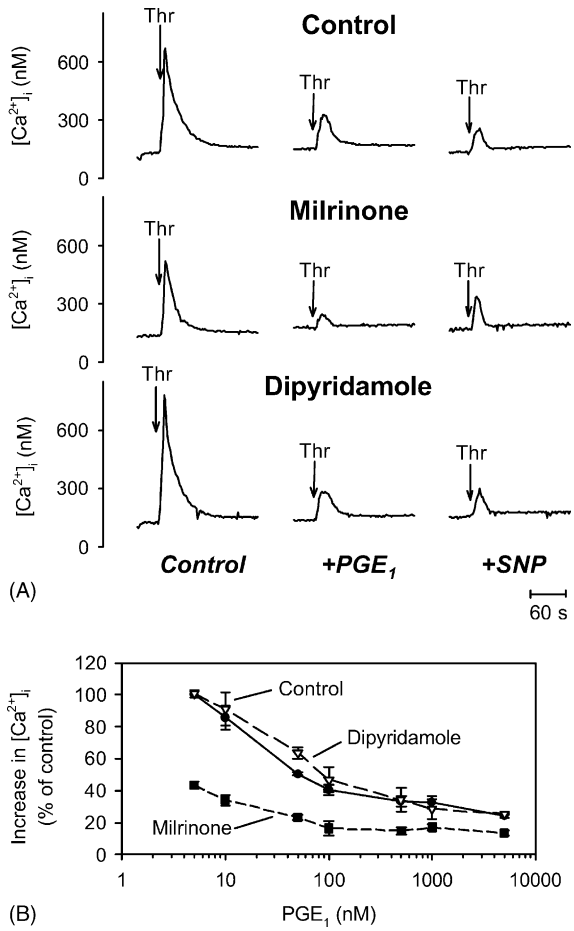

Fig. 1. Effect of milrinone and dipyridamole on thrombin-induced Ca^{2+} responses in platelets in the presence and absence of PGE_1 or SNP. Fura-2-loaded platelets were pre-incubated for 5 min without (control) or with milrinone (1 μM) or dipyridamole (20 μM), and then stimulated with thrombin (4 nM) (arrows). Where indicated platelets where also treated with PGE_1 (0.1 μM) or SNP (0.1 μM) at 2 min before thrombin stimulation. (A) Time-dependent increase of $[Ca^{2+}]_i$ after thrombin in the different conditions (data are representative for three or more independent experiments) and (B) dose-dependent effect of PGE_1 on maximal $[Ca^{2+}]_i$ increase after thrombin (mean ± S.E., $N = 3$).

milrinone and dipyridamole changed this response to $66 \pm 10\%$ ($P < 0.05$) and $118 \pm 5\%$ ($P > 0.05$), respectively (mean ± S.D., $N = 3$). In agreement with earlier results, stimulation of Gs by 0.1 μM PGE_1 lowered the thrombin-induced Ca^{2+} response from 623 ± 112 nM to 209 ± 21 nM ($P < 0.05$) (mean ± S.D., $N = 16$). The lowering effect of milrinone persisted at 0.1 μM PGE_1, and also at higher PGE_1 concentrations up to 5 μM (Fig. 1B). At 0.1 μM PGE_1, milrinone significantly reduced the maximal $[Ca^{2+}]_i$ rise ($P < 0.05$) induced by thrombin under this condition (Fig. 2A). Dipyridamole did not cause such a reducing effect. Dipyridamole also remained ineffective at high PGE_1 concentrations (Fig. 1B).

When a low concentration of the nitric oxide donor SNP (0.1 μM) was given to stimulate guanylate cyclase, this again reduced the $[Ca^{2+}]_i$ rise with thrombin from 623 ± 112 nM to 211 ± 38 nM (mean ± S.D., $N = 15$). In this case, neither milrinone nor dipyridamole influenced this SNP effect, giving $[Ca^{2+}]_i$ rises of $93 \pm 29\%$ and $79 \pm 28\%$ ($P > 0.05$) versus the condition with SNP only, respectively (mean ± S.D., $N = 3$).

To determine whether specifically PDE3 is involved in platelet Ca^{2+} signal generation, we tested the effect of a broader panel of PDE inhibitors on the maximal $[Ca^{2+}]_i$ rise (peak level) and the time-integral of the $[Ca^{2+}]_i$ rise. The latter parameter is an estimate of the total signaling capacity of thrombin [20]. The following compounds were used: IBMX and papaverine as general PDE inhibitors; EHNA as a PDE2 inhibitor; zardaverine, a PDE3/4 inhibitor, to compare with milrinone; Ro20-1724 as a PDE4 inhibitor; sildenafil and zaprinast as alternative PDE5 inhibitors [19,26,27]. Resting levels of $[Ca^{2+}]_i$ were not influenced by these compounds, up to maximally effective doses of 100 μM (data not shown). After 5-min of treatment, both the $[Ca^{2+}]_i$ peak and the integral with thrombin were reduced to 40–60% by the general PDE inhibitors IBMX and papaverine ($P < 0.05$) (Fig. 2A and B). Treatment with EHNA reduced only $[Ca^{2+}]_i$ peak with thrombin ($P < 0.05$), while treatment with zardaverine (PDE3 inhibition) reduced both Ca^{2+} response parameters again with 40–60% ($P < 0.05$). In contrast, the maximal Ca^{2+} response as well as the time integral were not significantly altered by PDE5 inhibition with dipyridamole, sildenafil or zaprinast ($P > 0.05$). Inhibition of PDE4 with Ro20-1724 was without effect (data not shown).

Upon low Gs stimulation with 0.1 μM PGE_1, the general and PDE3 inhibitors together with EHNA also significantly reduced the maximal thrombin-evoked Ca^{2+} response (Fig. 2A). With PGE_1 present, IBMX and papaverine also reduced the $[Ca^{2+}]_i$ time integrals ($P < 0.05$), while EHNA, milrinone, zardaverine and sildenafil had no significant effect ($P > 0.05$) (Fig. 2B). Dose–response curves (not shown) indicated that the reducing effects of IBMX and milrinone on thrombin-induced $[Ca^{2+}]_i$ rises had IC_{50} values of about 30 and 1 μM, respectively.

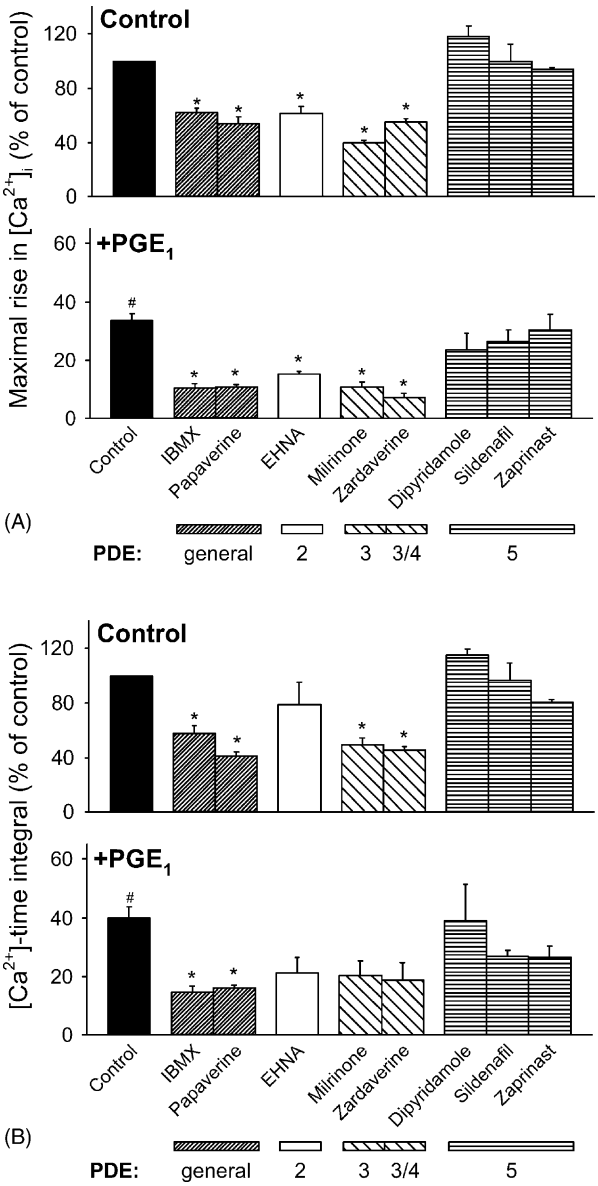

Fig. 2. Effect of different PDE inhibitors on thrombin-induced Ca^{2+} responses with or without PGE_1. Fura-2-loaded platelets were pre-incubated with vehicle (control) or PDE inhibitor for 5 min before stimulation with thrombin (4 nM). Where indicated, platelets were treated for 2 min with PGE_1 (0.1 μM). The PDE inhibitors were applied at 100 μM concentrations, except for milrinone (20 μM) and dipyridamole (20 μM). (A) Maximal rises in $[Ca^{2+}]_i$ with thrombin (percentage of control response). (B) Time integrals of rises in $[Ca^{2+}]_i$ with thrombin (percentage of control response). Data are mean values ± S.E. ($N = 3$–5). $^*P < 0.05$ vs. vehicle control, $^\#P < 0.05$ vs. control without PGE_1.

The monoclonal antibody PAC-1 [10] was used to determine the activation state of integrin αIIbβ3 on platelets in plasma. As binding to plasma proteins reduces the availability of various PDE inhibitors [28], these compounds were given at a high concentration of 200 μM. Platelets in plasma were stimulated with the PAR1 agonist SFLLRN instead of thrombin to prevent clotting. In the majority of the platelets, this caused PAC-1 binding, which was completely reversed by cAMP-elevating PGE_1 (Fig. 3A). General PDE inhibitors (IBMX and papaverine) as well as

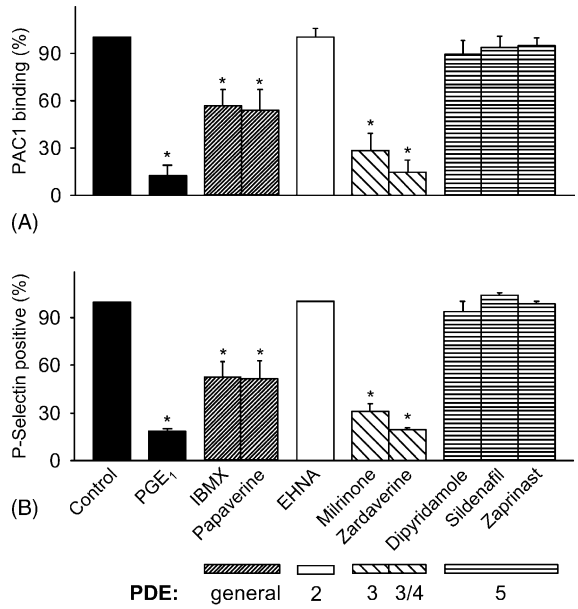

Fig. 3. Effect of different PDE inhibitors on SFLLRN-induced integrin αIIbβ3 activation and P-selectin exposure. PRP was pre-incubated with indicated PDE inhibitor for 5 min before platelet stimulation with SFLLRN (10 μM) in the absence of stirring. Inhibitors were used at 200 μM concentration, except for milrinone (20 μM) and dipyridamole (100 μM). Alternatively, PRP was pre-incubated with PGE_1 (1 μM). Samples were taken for flow-cytometric analysis of αIIbβ3 activation or P-selectin exposure using PAC-1 (A) or anti-CD62P (B) antibody, respectively. Data are percentages of positive platelets compared to control condition (100%). Mean values ± S.E. ($N = 3$–5). $^{*}P < 0.05$ vs. vehicle control.

PDE3 inhibitors (milrinone and zardaverine) reversed the SFLLRN-induced integrin activation ($P < 0.05$). Addition of EHNA or inhibition of PDE5 (dipyridamole, sildenafil and zaprinast) was without effect ($P > 0.05$).

Surface expression of P-selectin during exocytosis was measured as a downstream event of the platelet Ca^{2+} signal. In the presence of plasma, only the general and type-3 PDE inhibitors significantly suppressed SFLLRN-induced expression of P-selectin ($P < 0.05$) (Fig. 3B). Together, these results indicate that only PDE3 inhibition reduced thrombin receptor-mediated integrin αIIbβ3 activation and exocytosis.

3.2. Platelet inhibition due to PDE blockade mediated by cAMP but not cGMP

If PDE3 is the main PDE modulating platelet Ca^{2+} signal generation and integrin affinity regulation, it is expected that cAMP rather than cGMP controls these processes. This was verified by determining the levels of cyclic nucleotides in measurements parallel to Ca^{2+} responses. Table 1 shows that treatment of platelets with general PDE inhibitors (IBMX, papaverine) or PDE3 inhibitors (milrinone and zardaverine, the latter at borderline significance) raised the cAMP level. In the presence of low, 0.1 μM PGE_1, elevating cAMP by itself, these compounds caused a further increase in cAMP. In contrast, treatment with PDE5 inhibitors (dipyridamole, sildenafil and zaprinast) did not influence cAMP in the absence or presence of PGE_1. Furthermore, SNP led to a significant, twofold rise in cAMP concentration, which was not further increased with PGE_1. These results thus indicated that inhibition of PDE3 alone increased the platelet cAMP concentration, in a way potentiated by low Gs stimulation with PGE_1.

With respect to cGMP, this cyclic nucleotide was increased with the PDE5 inhibitors (borderline significant with dipyridamole) and with the general PDE inhibitor IBMX (borderline significance) (Table 1). Stimulation of guanylate cyclase by low, 0.1 μM SNP also resulted in an about twofold stimulated cGMP level. In combination with SNP, general PDE inhibition (papaverine) and PDE5

Table 1
Effect of PDE inhibitors on platelet cAMP and cGMP levels

Addition (μM)	Type	cAMP		cGMP	
		$-PGE_1$	$+PGE_1$	−SNP	+SNP
None (control)		1.00	6.74 ± 0.40$^{\#}$ (9)	1.00	1.73 ± 0.21$^{\#}$ (8)
IBMX (100)	General	1.40 ± 0.05* (7)	12.67 ± 1.71* (5)	2.18 ± 0.11 (3)	4.36 ± 0.93 (5)
Papaverine (100)	General	2.02 ± 0.10* (9)	15.30 ± 0.57* (4)	2.53 ± 0.36 (4)	7.02 ± 0.56* (4)
EHNA (100)	2	1.43 ± 0.14 (8)	7.45 ± 0.46 (4)	1.49 ± 0.14 (4)	2.18 ± 0.12 (3)
Milrinone (20)	3	2.33 ± 0.14* (10)	13.42 ± 1.92* (5)	1.06 ± 0.08 (5)	2.20 ± 0.19 (7)
Zardaverine (100)	3/4	1.53 ± 0.07 (9)	11.20 ± 1.30 (4)	2.52 ± 0.12 (4)	6.83 ± 0.69* (6)
Dipyridamole (20)	5	0.95 ± 0.05 (11)	6.22 ± 1.65 (3)	2.46 ± 0.21 (5)	5.95 ± 0.63* (5)
Zaprinast (100)	5	1.15 ± 0.08 (11)	4.09 ± 0.31 (4)	10.90 ± 2.44* (4)	14.65 ± 1.15* (4)
Sildenafil (100)	5	0.94 ± 0.07 (11)	7.51 ± 0.69 (3)	8.34 ± 0.70* (4)	15.48 ± 1.79* (5)
SNP (0.1)		2.00 ± 0.18* (9)	7.19 ± 0.17 (4)	–	1.73 ± 0.21$^{\#}$ (8)
PGE_1 (0.1)		–	6.74 ± 0.40$^{\#}$ (9)	n.d.	n.d.

Platelets were pre-incubated with indicated PDE inhibitor (5 min) with or without PGE_1 (0.1 μM) or SNP (0.1 μM). Data of cAMP and cGMP levels are compared to control condition without inhibitor, at which cAMP and cGMP levels were 2.59 ± 0.17 pmol/10^8 platelets ($N = 12$) and 0.56 ± 0.12 pmol/10^8 platelets ($N = 8$), respectively. Mean ± S.E. (N).

$^{*}P < 0.05$ vs. vehicle control.

$^{\#}P < 0.05$ vs. absence of PGE_1 or SNP.

inhibition (dipyridamole, zaprinast, sildenafil) further raised the cGMP level, with zaprinast and sildenafil being most active. A similar, minor increase in cGMP by dipyridamole alone but high increase upon simultaneous guanylate cyclase activation has also been observed by others [29]. Typically, the type-3/4 inhibitor zardaverine also caused cGMP elevation in this case.

It has been reported that inositol 1,4,5-trisphosphate-mediated Ca^{2+} mobilization, following Gq-coupled receptor activation, is antagonized by elevation of cAMP and cGMP [9,30]. The current results allow direct comparison of the cyclic nucleotides levels and the inositoltrisphosphate-mediated Ca^{2+} response with thrombin. We found a steep inverse relation between cAMP (Fig. 4A) but not cGMP (Fig. 4B) concentration and the thrombin-induced Ca^{2+} peak in platelets, regardless of whether the cells were pretreated with PGE_1 or SNP or left untreated. This indicated that the cAMP level, as influenced by the PDE inhibitors, was a main determinant of the Ca^{2+} response

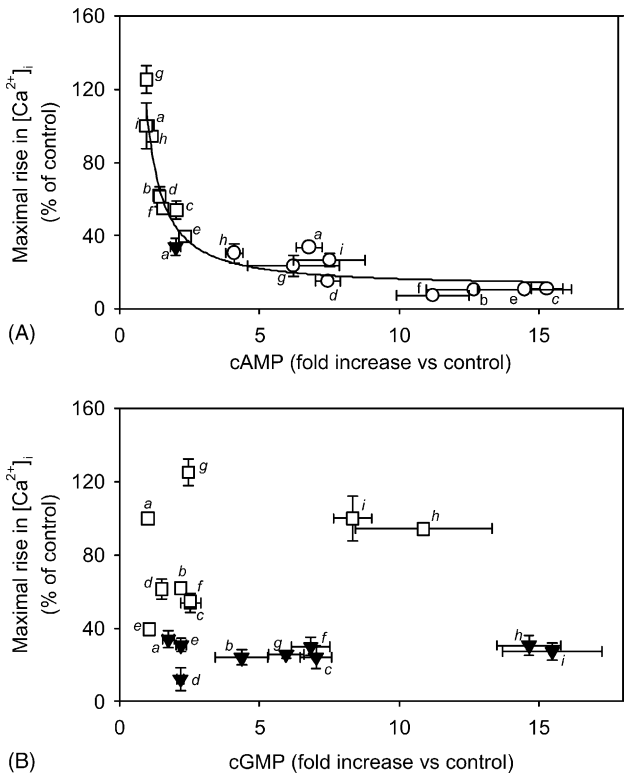

Fig. 4. Relation between thrombin-induced Ca^{2+} mobilization and cyclic nucleotide levels in platelets pre-incubated with PDE inhibitors. Fura-2-loaded platelets were pre-incubated with vehicle or PDE inhibitor for 5 min, after which rises in $[Ca^{2+}]_i$ in response to thrombin (4 nM) were measured. Where indicated, platelets were also treated for 2 min with PGE_1 (0.1 μM) or SNP (0.1 μM). Concentrations of PDE inhibitors were as in Fig. 2. In parallel incubations, samples were taken before stimulation with thrombin to measure cytosolic levels of cAMP and cGMP. (A) Plot of Ca^{2+} response with thrombin vs. cAMP level. (B) Plot of Ca^{2+} response with thrombin vs. cGMP level. Code: *a*, vehicle (no PDE inhibitor); *b*, IBMX; *c*, papaverine; *d*, EHNA; *e*, milrinone; *f*, zardaverine; *g*, dipyridamole; *h*, zaprinast; *i*, sildenafil; (□), control condition (no pretreatment); (○), PGE_1 pretreatment; (▼), SNP pretreatment. Mean ± S.E. ($N = 4$–6).

with thrombin. Because αIIbβ3 activation is influenced by PDE inhibitors in a similar way as the Ca^{2+} signal (compare Figs. 2 and 3), the same inverse relation is likely to hold for the cAMP (but not cGMP) level and αIIbβ3 affinity regulation.

Further confirmation for the crucial role of PDE3 in cAMP modulation was obtained from measurements, where platelets were treated with sub-maximal doses of milrinone (0.5 μM). In this case, treatment for 5 min altered neither the cAMP level nor the thrombin-induced Ca^{2+} response, whereas 60-min treatment resulted in a 1.5-fold lowering of the thrombin-induced Ca^{2+} response, respectively, and an 161% increase in cAMP (means of two experiments, data not shown). In contrast, a high dose (100 μM) and prolonged incubation (60 min) with sildenafil was needed to detect a reducing effect (−20%) of this compound on the Ca^{2+} response. Both the altered Ca^{2+} response and cAMP level by PDE3 inhibition (45-min incubation with milrinone) were normalized after wash of the platelets (responsiveness to thrombin 109 ± 8% of control, $P > 0.05$). This indicates nearly complete reversibility of the action of the PDE inhibitors.

The possibility was investigated that adenosine derived from platelets or produced by apyrase action interferes with the effect of PDE3 inhibitors [31]. However, pretreatment of the platelets with adenosine deaminase (2 U/ml) did not influence the thrombin-induced Ca^{2+} responses (data not shown).

3.3. Suppression of platelet aggregation and thrombin generation in coagulating plasma by inhibition of PDE3 but not of PDE5

We subsequently measured aggregation and thrombin formation as functional platelet responses, being dependent on both integrin αIIbβ3 activation and Ca^{2+} signaling [32]. In PRP, both non-selective PDE inhibitors (IBMX and papaverine) and PDE3 inhibitors (milrinone and zardaverine) completely suppressed the aggregation of platelets induced by PAR1 agonist, SFLLRN (Fig. 5). While EHNA was without effect, PDE5 inhibition with dipyridamole, zaprinast or sildenafil caused reversible aggregation in PRP from the majority of the donors (Fig. 5), but was without effect on PRP from some donors (not shown).

In measurements of platelet-dependent thrombin generation in coagulating PRP, both general PDE inhibitors (IBMX and papaverine) and type-3 PDE inhibitors (milrinone and zardaverine) greatly suppressed this response (Fig. 6A and B). Only the reducing effect of milrinone was statistically significant under this condition ($P < 0.05$). The inhibitor effects were more pronounced in the presence of low 0.01 μM PGE_1 (Fig. 6B), in which case the effects of all general (IBMX and papaverine) and type-3 PDE inhibitors (milrinone and zardaverine) were significant ($P < 0.05$). Again, EHNA (PDE2 inhibition) and the

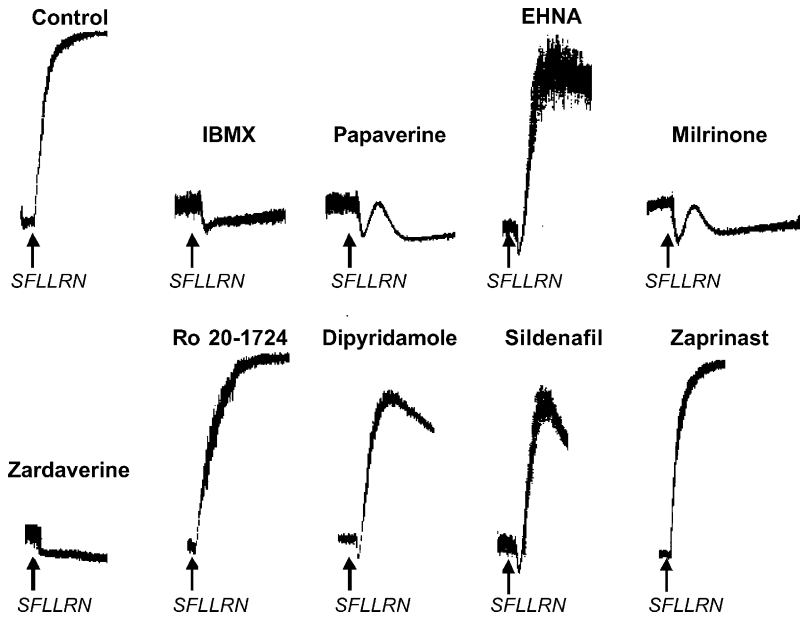

Fig. 5. Effect of different PDE inhibitors on SFLLRN-induced platelet aggregation. PRP was incubated with indicated PDE inhibitor for 5 min, before platelet stimulation with SFLLRN (10 μM) under stirring (900 rpm). Concentrations of inhibitors were as in Fig. 3. Aggregation traces are representative for three to five independent experiments.

PDE5 inhibitors dipyridamole, sildenafil and zaprinast were ineffective.

4. Discussion

In the 1970s, inhibitors of PDEs have been investigated for potential clinical use in antiplatelet treatment [17,33]. In most studies, authors have examined inhibiting effects of these drugs on platelets mostly under conditions of elevated levels of these cyclic nucleotides, e.g. in platelets treated with prostaglandin or nitric-oxide donor. Here, we present new evidence that PDE3 inhibition increases the cAMP level also under basal conditions, while PDE5 inhibition increases the 'basal' cGMP level, i.e. without exogenous stimulation of Gs/adenylate cyclase or guanylate cyclase. With respect to cAMP, we find that: (1) cAMP accumulation can be achieved by inhibition of PDE3 without ligand stimulation of Gi or Gs; (2) concomitant PDE3 inhibition and Gs stimulation with low PGE_1 further increases cAMP accumulation; (3) PDE3 inhibition suppresses the Ca^{2+}, secretion, aggregation and procoagulant responses of platelets in a way compatible with the increased cAMP level. On the other hand, PDE5 inhibition results in an increased cGMP concentration particularly with zaprinast or sildenafil and, less potently, with dipyridamole. However, the altered cGMP did not alter platelet responses. Together, these findings demonstrate that PDE3 and PDE5 function to keep cAMP and cGMP, respectively, at relatively low levels, even in resting platelets, and thus reverse the non-zero basal activities of adenylate and guanylate cyclase. In combination with the reported evidence that resting platelets have a slightly increased cAMP level [11], we can conclude that the platelet cAMP level is in continuous turnover even in the absence of receptor-mediated Gs stimulation. As platelets are known to produce nitric oxide [34], it is possible that this autocrine production is responsible for the basal, non-zero guanylate cyclase activity.

The present findings with general PDE inhibitors (IBMX and papaverine) and type-3 PDE inhibitors (milrinone and zardaverine) point to a steep inverse relationship between cAMP level and the thrombin-induced Ca^{2+} response. Thus, small increases in cAMP above the basal level resulted in a potent reduction in Ca^{2+} mobilization (Fig. 4). A similar, steep relation has been observed before in experiments where cAMP was elevated with PGE_1, and there it was attributed to protein kinase A phosphorylation of inositol 1,4,5-trisphosphate receptors and subsequent reduction of Ca^{2+}-induced Ca^{2+} release of these receptors [9,11]. This high sensitivity of Ca^{2+} mobilization to small changes in cAMP may explain controversies in the literature with respect to the mechanism of action of PDE inhibitors. For instance, Manns et al. have reported that 10-s treatment of platelets with EHNA and to a lesser degree with milrinone, each at 100 μM, suppressed agonist-induced Ca^{2+} responses, whereas high, millimolar concentrations of these compounds were required for a substantial increase in cAMP after 1 min [35]. Yet, these authors showed that the short-term treatment resulted in cAMP-mediated VASP phosphorylation, which is in agreement with the present notion that even slight increases in cAMP are sufficient to modulate platelet activation. There is limited supportive evidence in earlier papers that compounds like IBMX and milrinone reduce platelet aggregation, particularly when indirect thromboxane effects were

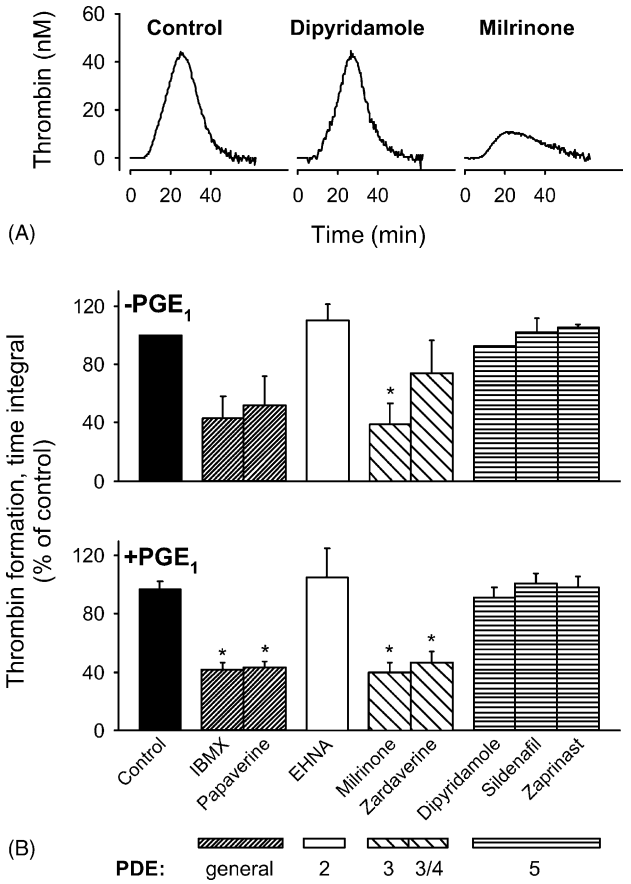

Fig. 6. Suppression of thrombin generation in coagulating PRP by general and type-3 PDE inhibitors. Citrated PRP, pre-incubated with indicated PDE inhibitor, was triggered with tissue factor (0.5 pM) and $CaCl_2$ (16.6 mM). PDE inhibitors (±0.01 μM PGE_1) were used at concentrations as in Fig. 3. Thrombin generation was determined from fluorescence accumulation due to cleavage of Z-GGR-AMC. (A) Representative curves of thrombin generation in the absence (control) or presence of milrinone (20 μM) or dipyridamole (100 μM). (B) Time integrals of generated thrombin (areas under the curve) relative to vehicle control. Data are means ± S.E. ($N = 5$; EHNA, $N = 2$). $^*P < 0.05$ vs. vehicle control.

blocked [24,36,37], or reduce platelet secretion [38], but these effects have not been compared with cyclic nucleotide levels. Concerning integrin activation, it should be noted that cAMP modulation as such is not sufficient to trigger these responses, even not when stimulated via Gi/z and G12/13 [5,6,39].

The current data indicate that especially PDE3 has a role in platelet activation under conditions of non-increased cAMP and cGMP levels. The cGMP-stimulated PDE2 has been proposed to function mainly under conditions of high cGMP [12]. In agreement with this notion, we measured inhibiting effects of EHNA (PDE2 inhibitor) on Ca^{2+} responses (Fig. 2). This reduction was accompanied with a small, insignificant increase in cAMP level (Table 1), which thus may point to contribution of PDE2 in maintaining low cAMP.

Recent findings by Li et al. have challenged the traditional concept that cGMP like cAMP acts to inhibit platelet function [7,12]. It has been proposed that early activation of the cGMP/protein kinase G pathway has an initial stimulatory role on platelet aggregation [40]. The present data indicate that cGMP elevation per se is not inhibitory for various platelet responses. Thus, PDE5 inhibitors zaprinast and sildenafil, after 5-min treatment, raised cGMP but not cAMP levels, but were unable to block activation of platelets. The cGMP-elecating effects of these compounds was potentiated by simultaneous activity of guanylate cyclase with SNP. Inhibition of PDE5 with dipyridamole caused similar but less potent rises in cGMP but, interestingly, similar cGMP changes as general PDE inhibition with IBMX or papaverine.

In contrast, 5-min treatment with SNP, raising cGMP and also cAMP via PDE3 activity, consistently blocked platelet Ca^{2+} responses (Fig. 2) and aggregation (see [25,41]). In addition, we did not find a clear relation between the cGMP level and Ca^{2+} responses.

The finding of continuing cAMP formation in apparently resting cells is probably not confined to platelets, as many cells have non-zero levels of this cyclic nucleotide in the absence of adenylate cyclase-stimulating agonists. We do not yet know the biochemical mechanism responsible for the 'basal' adenylate cyclase activity in resting platelets. A possibility is involvement of G-protein coupled receptors, e.g. Gs-stimulating (orphan) receptors with partial activity in the absence of ligand, or variable activity of receptors coupled to Gi-family proteins, as these are abundantly present in the platelet membrane. However, we can not exclude the possibility that other autocrine or environmental factors also contribute to set the 'basal' adenylate cyclase tone.

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References

[1] Daniel JL, Dangelmaier C, Smith JB. Evidence for a role for tyrosine phosphorylation of phopholipase Cγ2 in collagen-induced platelet cytosolic calcium mobilisation. Biochem J 1994;302:617–22.
[2] Offermanns S, Toombs CF, Hu Y, Simon MI. Defective platelet activation in $G\alpha_q$-deficient mice. Nature 1997;389:183–6.
[3] Sage SO. Calcium entry mechanisms in human platelets. Exp Physiol 1997;82:807–23.
[4] Heemskerk JWM. Calcium and platelets. In: Pochet R, Donato R, Haiech J, Heinzmann C, Gerke V, editors. The molecular basis of calcium action in biology and medicine. The Hague: Kluwer Academic Publications; 2000. p. 45–71.
[5] Yang J, Wu J, Kowalska MA, Dalvi A, Prevost N, O'Brien PJ, et al. Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. Proc Natl Acad Sci USA 2000;97:9984–9.
[6] Dorsam RT, Kim S, Ji J, Kunapuli SP. Coordinated signaling through both G12/13 and Gi pathways is sufficient to activate GPIIb/IIIa in human platelets. J Biol Chem 2002;277:47588–95.

[7] Schwartz UR, Walter U, Eigenthaler M. Taming platelets with cyclic nucleotides. Biochem Pharmacol 2001;62:1153–61.
[8] Den Dekker E, Gorter G, Heemskerk JWM, Akkerman JWN. Development of platelet inhibition by cAMP during megakaryocytopoiesis. J Biol Chem 2002;277:29321–9.
[9] Cavallini L, Coassin M, Borean A, Alexandre A. Prostacyclin and sodium nitroprusside inhibit the activity of the platelet inositol 1,4,5-trisphosphate receptor and promote its phosphorylation. J Biol Chem 1996;271:5545–51.
[10] Shattil SJ, Kashiwagi H, Pampori N. Integrin signaling, the platelet paradigm. Blood 1998;91:2645–57.
[11] Keularts IMLW, van Gorp RMA, Feijge MAH, Vuist WMJ, Heemskerk JWM. α_{2A}-Adrenergic receptor stimulation potentiates calcium release in platelets by modulating cAMP levels. J Biol Chem 2000; 275:1763–72.
[12] Haslam RJ, Dickinson NT, Jang EK. Cyclic nucleotides and phosphodiesterases in platelets. Thromb Haemost 1999;82:412–23.
[13] Conti M, Jin SLC. The molecular biology of cyclic nucleotide phosphodiesterases. In: Progress in nucleic acid research and molecular biology, vol. 63. New York: Academic Press; 2000. p. 1–38.
[14] Halbrügge M, Walter U. The regulation of platelet function by protein kinases. In: Huang CK, Sha'afi RI, editors. Protein kinases in cell function. Boca Raton, FL: CRC Press; 1993. p. 245–98.
[15] Dickinson NT, Jang EK, Haslam RJ. Activation of cGMP-stimulated phosphodiesterase by nitroprusside limits cAMP accumulation in human platelets: effects on platelet aggregation. Biochem J 1997; 323:371–7.
[16] Li Z, Xi X, Gu M, Feil R, Ye RD, Eigenthaler M, et al. A stimulatory role for cGMP-dependent protein kinase in platelet activation. Cell 2003;112:77–86.
[17] Haslam RJ, Davidson MM, Davies T, Lynham JA, McClenaghan MD. Regulation of blood platelet function by cyclic nucleotides. Adv Cyclic Nucl Res 1978;9:533–52.
[18] Picano E, Abbracchio MP. European stroke prevention study-2: serendipitous demonstration of neuroprotection induced by endogenous adenosine accumulation? Trends Pharmacol Sci 1998;19:14–6.
[19] Wallis RM, Corbin JD, Francis SH, Ellis P. Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. Am J Cardiol 1999;83: 3C–12C.
[20] Heemskerk JWM, Feijge MAH, Henneman L, Rosing J, Hemker HC. The Ca^{2+}-mobilizing potency of a-thrombin and thrombin-receptor-activating peptide on human platelets. Eur J Biochem 1997;249: 547–55.
[21] Feijge MAH, van Pampus ECM, Lacabaratz-Porret C, Hamulyak K, Lévy-Toledano S, Enouf J, et al. Inter-individual variability in Ca^{2+} signalling in platelets from healthy volunteers: effects of aspirin and relationship with expression of endomembrane Ca^{2+}-ATPases. Br J Haematol 1998;102:850–9.
[22] Hemker HC, Ramjee M, Wagenvoord R, Béguin S. The thrombogram: monitoring thrombin generation in platelet rich plasma. Thromb Haemost 2000;83:589–91.
[23] Hemker HC, Béguin S. Thrombin generation in plasma: its assessment via the endogenous thrombin potential. Thromb Haemost 1995;74: 134–8.
[24] Barradas MA, Jagroop A, O'Donoghue S, Jeremy JY, Mikhailidis DP. Effect of milrinone in human platelet shape change, aggregation and thromboxane A_2 synthesis: an in vitro study. Thromb Res 1993;71: 227–36.
[25] Gresele P, Zoja C, Deckmyn H, Arnout J, Vermylen J, Verstraete M. Dipyridamole inhibits platelet aggregation in whole blood. Thromb Haemost 1983;50:852–6.
[26] Beavo JA. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol Rev 1995;75:725–48.
[27] Soderling SH, Bayuga SJ, Beavo JA. Identifiaction and characterization of a novel family of cyclic nucleotide phosphodiesterases. J Biol Chem 1998;273:15553–8.
[28] Szebeni J, Weinstein JN. Dipyridamole binding to proteins in human plasma and tissue culture media. J Lab Clin Med 1991;117:485–92.
[29] Wu CC, Ko FN, Teng CM. Inhibition of platelet adhesion to collagen by cGMP-elevating agents. Biochem Biophys Res Commun 1997; 231:412–6.
[30] Tertyshnikova S, Yan X, Fein A. cGMP inhibits IP_3-induced Ca^{2+} release in intact rat magakaryocytes via cGMP- and cAMP-dependent protein kinases. J Physiol 1998;512:89–96.
[31] Sun B, Le SN, Lin S, Fong M, Guertin M, Liu Y, et al. New mechanism of action of cilostazol: interplay between adenosine and cilostazol in inhibiting platelet activation. J Cardiovasc Pharmacol 2002;40: 577–85.
[32] Heemskerk JWM, Bevers EM, Lindhout T. Platelet activation and blood coagulation. Thromb Haemost 2002;88:186–93.
[33] Mills DC, Smith JB. The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3′:5′-cyclic monophosphate in platelets. Biochem J 1971;121:185–96.
[34] Chen L, Salafranca MN, Mehta JL. Cyclooxygenase inhibition decreases nitric oxide synthase activity in human platelets. Am J Physiol 1997;273:H1854–9.
[35] Manns JM, Brennan KJ, Colman RW, Sheth SB. Differential regulation of human platelet responses by cGMP inhibited and stimulated cAMP phosphodiesterases. Thromb Haemost 2002;87:873–9.
[36] Asano T, Ochiai Y, Hidaka H. Selective inhibition of separated forms of human platelet cyclic nucleotide phosphodiesterase by platelet aggregation inhibitors. Mol Pharmacol 1977;13:400–6.
[37] Smith JB. Effect of thromboxane synthetase inhibitors on platelet function: enhancement by inhibition of phosphodiesterase. Thromb Res 1982;28:477–85.
[38] Roevens P, De Chaffoy de Courcelles D. Cyclic AMP-phosphodiesterase IIIA1 inhibitors decrease cytosolic Ca^{2+} concentration and increase the Ca^{2+} content of intracellular storage sites in human platelets. Biochem Pharmacol 1993;45:2279–82.
[39] Kim S, Foster C, Lecchi A, Quinton TM, Prosser DM, Jin J, et al. Protease-activated receptors 1 and 4 do not stimulate Gi signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of Gi signaling. Blood 2002;99:3629–36.
[40] Li Z, Ajdic J, Eigenthaler M, Du X. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. Blood 2003;101:4423–9.
[41] Best LC, McGuire MB, Jones PB, Holland TK, Martin TJ, Preston FE, et al. Mode of action of dipyridamole on human platelets. Thromb Res 1979;16:367–79.