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European Journal of Pharmacology 517 (2005) 149 – 157

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Cross-talk between adenosine and the oxatriazole derivative GEA 3175 in platelets

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Received 3 March 2005; received in revised form 12 May 2005; accepted 20 May 2005
Available online 15 June 2005

Abstract

We examined the interplay between adenosine and the nitric oxide (NO)-containing oxatriazole derivative GEA 3175 in human platelets. The importance of cyclic guanosine 3′5′-monophosphate (cGMP)-inhibited phosphodiesterases (PDEs) was elucidated by treating the platelets with adenosine combined with either GEA 3175 or the PDE3-inhibitor milrinone. The drug combinations provoked similar cyclic adenosine 3′5′-monophosphate (cAMP) responses. On the contrary, cGMP levels were increased only in GEA 3175-treated platelets. Both drug combinations reduced P-selectin exposure, platelet adhesion and fibrinogen-binding. However, adenosine together with GEA 3175 was more effective in inhibiting platelet aggregation and ATP release. Thrombin-induced rises in cytosolic Ca^{2+} were suppressed by the two drug combinations. Adenosine administered with GEA 3175 was, however, more effective in reducing Ca^{2+} influx.

In conclusion, the interaction between adenosine and GEA 3175 involves cGMP-mediated inhibition of PDE3. The results also imply that inhibition of Ca^{2+} influx represent another cGMP-specific mechanism that enhances the effect of adenosine.

Keywords: Adenosine; Calcium; cAMP; cGMP; GEA 3175; Milrinone; Nitric oxide; Phosphodiesterases; Platelets

1. Introduction

Adenosine, released from cells via nucleoside transport proteins or produced in the extracellular compartment by ecto 5′-nucleotidases, is an endogenous inhibitor of platelets. The basic mechanism of action of adenosine involves binding to membrane receptors at the surface of the platelet. Several subtypes of G-protein-coupled receptors with affinity for adenosine have been characterised and they are designated adenosine A_1-A_{2A}-A_{2B}-, and A_3-receptors (Ralevic and Burnstock, 1998). Binding studies have indicated the existence of two different adenosine-binding sites on platelet plasma membrane, but there is no conclusive evidence that platelets express receptor subtypes other than the adenosine A_{2A}-receptors (Varani et al., 1996). Occupancy of adenosine A_{2A}-receptors leads to activation of membrane-bound adenylyl cyclase and increased synthesis of cyclic adenosine 3′5′-monophosphate (cAMP). This cyclic nucleotide activates protein kinase A (PKA) resulting in increased serine/threonine-specific phosphorylation of a great number of platelet proteins. From a cell physiological point of view, elevation of cAMP is associated with suppression of all platelet functional responses.

Nitric oxide (NO) also exhibits anti-platelet actions. It is well established that NO stimulates cytosolic guanylyl cyclase, resulting in increases in platelet cyclic guanosine 3′5′-monophosphate (cGMP) content. In common with the cAMP signalling system, cGMP binds to and activates a serine/threonine-specific kinase termed protein kinase G (PKG). PKA and PKG have some common sites of action in platelets. For example, the Ca^{2+}-channel receptor for inositol 1,4,5-trisphosphate and the 46/50 kDa vasodilator-stimulated

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doi:10.1016/j.ejphar.2005.05.019

phosphoprotein (VASP) are phosphorylated by both PKG and PKA (Cavallini et al., 1996; Aszodi et al., 1999). Conversely, several findings suggest that PKA and PKG exert separate mechanisms of action in platelets. For instance, inhibition of phospholipase C_γ (PLC_γ) and activation of Ca^{2+}-ATPases, located on the dense tubular system, resulting in reduction of cytosolic Ca^{2+} is attributed to cAMP, but not to cGMP (Peterson and Lapetina, 1994; Dean et al., 1999). In platelets, cGMP also regulates phosphodiesterase (PDE) activity. Isoenzymes of PDEs designated PDE2 (allosteric activation by cGMP, hydrolyses cAMP and cGMP), PDE3 (allosteric inhibition by cGMP, hydrolyses cAMP and cGMP), and PDE5 (regulated by phosphorylation, hydrolyses cGMP) have been identified in platelets (Haslam et al., 1999). Consequently, cGMP may both enhance and restrict the accumulation of cAMP in platelets. Taken together, there is a large body of evidence for an intriguing interplay between the cAMP and the cGMP signalling pathways in platelets. Indeed, it has been documented that simultaneous treatment with NO or NO-donors and cAMP-elevating compounds synergistically suppress platelet adhesion, secretion and aggregation (Radomski et al., 1987; Grenegård et al., 1996). In this regard, it has been proposed that the cGMP-mediated inhibition of PDE3 is the main molecular basis of synergy (Maurice and Haslam, 1990).

From a pharmacological perspective, it has been shown that externally applied NO-donors, such as S-nitrosothiols and sydnonimine derivatives inhibit platelet responses (Salas et al., 1994; Keh et al., 2003). On the other hand, platelets are much less sensitive to clinically used organic nitrates, like nitroglycerin (Gerzer et al., 1988). One reason for that is the incapability of platelets to metabolise the drugs, resulting in impaired release of free NO molecules. We have previously characterised the pharmacological properties of the NO-containing oxatriazole derivative GEA 3175. Interestingly, GEA 3175, in the μM concentration range, did not liberate a detectable amount of NO but still produced a long-lasting inhibition of platelet responses (Asplund Persson et al., 2004). Furthermore, in an earlier study, we found that adenosine and GEA 3175 interacted synergistically to inhibit platelet aggregation (Grenegård et al., 1996). This phenomenon presumably involves cGMP-mediated inhibition of class 3 PDE activity. In fact, PDE3 is believed to play a pivotal role in the regulation of platelets responsiveness and inhibitors of PDE3 may represent an important class of novel, effective antiplatelet agents (Manns et al., 2002; Feijge et al., 2004).

One main aim in the present study was to clarify the importance of the enhanced cAMP response obtained when adenosine and GEA 3175 are administered together. Furthermore, the intention was to identify or exclude a role for other cGMP-specific mechanisms, besides PDE-inhibition, participating in the interplay between adenosine and the oxatriazole derivative GEA 3175. In order to clarify these aims, the class 3 PDE-inhibitor milrinone replaced GEA 3175 in some experimental designs. The inhibitory actions of adenosine combined with either GEA 3175 or milrinone were thoroughly characterised and compared.

2. Materials and methods

2.1. Isolation of human platelets

Human blood was collected from healthy volunteers and immediately mixed with an acid-citrate-dextrose solution (5 volumes blood and 1 volume acid-citrate-dextrose solution) composed of 85 mM Na-citrate, 71 mM citric acid and 111 mM glucose. The blood was centrifuged for 20 min at 220 $\times g$ to obtain platelet-rich plasma. Acetylsalicylic acid (100 μM) and apyrase (1 U/ml) were added to the platelet-rich plasma to prevent platelet activation by thromboxane A_2 and ADP during the isolation procedure. The platelet-rich plasma was then centrifuged for 20 min at 480 $\times g$ to obtain a pellet of platelets. The supernatant was removed and the platelets were gently resuspended in an HEPES buffer (pH 7.4) composed of 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES, 1 U/ml apyrase and 10 mM glucose. The platelet suspensions were stored in plastic tubes at room temperature and were used within 3 h after preparation. Extracellular Ca^{2+} concentration was adjusted to 1 mM immediately before each measurement.

2.2. Experimental designs

In the experimental protocols, platelets were generally treated with 10 μM of adenosine, 10 μM of GEA 3175, or a combination of the two compounds for 2 min. Thereafter, the platelets were activated with thrombin (0.1 U/ml). The concentration of an activator is certainly of importance when characterising the actions and effectiveness of different inhibitory compounds. Initial dose–response series revealed that thrombin (0.001–0.3 U/ml) induced platelet aggregation, ATP release and rises in $[Ca^{2+}]_i$ with EC_{50} values of, respectively, 0.0056 U/ml (95% confidence limit: 0.0048–0.0065), 0.056 U/ml (0.040–0.079), and 0.033 U/ml (0.020–0.054). These results show that the dose of thrombin commonly used in the present investigation (i.e., 0.1 U/ml) is sufficient to induce powerful intracellular and cellular responses in platelets.

The concentration of adenosine and GEA 3175 and the length of incubation are based on our earlier studies. The main conclusion from these studies is that 10 μM of adenosine or GEA 3175 is sufficient to produce maximal effect of the drugs under our experimental conditions. For instance, we have shown that maximal cGMP-elevating and cytosolic Ca^{2+}-reducing capacities are achieved around a concentration of 1 to 10 μM of GEA 3175 (Grenegård et al., 1996, Asplund Persson et al., 2004). Similarly, adenosine-induced inhibition of $[Ca^{2+}]_i$ is maximal in the low μM concentration range (Grenegård et al., 1996).

In the present study, the inhibitor of cGMP-inhibited class 3 PDEs milrinone was used. The IC_{50}-value for milrinone has been estimated to be about 0.1–0.3 μM (Harrison et al., 1986; Maurice et al., 2003). Based on that milrinone was tested in the concentration range between 0.03 to 10 μM (data not shown). These preliminary experiments revealed that milrinone from 0.3 up to 3 μM had no intrinsic effect, but markedly enhanced the actions of adenosine. It is to be noticed that higher concentrations of milrinone directly affected platelet responsiveness but also

influenced on some experimental techniques, like the fluorescent properties of fura-2. For these reasons platelets were exposed to 1 μM of milrinone for 5 min. After additional 3 min the platelets were exposed to adenosine and finally stimulated with thrombin.

In some experimental designs (i.e., aggregation (Section 2.3) and ATP release (Section 2.6)), adenosine was replaced by forskolin. This compound directly activates adenylyl cyclase. Forskolin (10 nM) was incubated for 2 min in the absence or presence of GEA 3175 or milrinone. Thereafter, the platelets were stimulated by adding thrombin.

GEA 3175, forskolin and milrinone were dissolved in dimethyl sulfoxide; the final concentration of dimethyl sulfoxide in the cell suspensions did not exceed 0.2%. The solution of GEA 3175 was always kept in the dark.

2.3. Recording of platelet aggregation

Aliquots (0.5 ml) of platelets (2.5×10^8/ml) were preincubated at 37 °C for 2 min and then exposed to adenosine, forskolin, GEA 3175, milrinone alone and in combinations. Platelet aggregation was induced by adding thrombin and changes in light transmission were recorded using a Chronolog Dual Channel lumi-aggregometer (Model 560, Chrono-Log, Haverston, PA, U.S.A).

2.4. Analysis of platelet adhesion to fibrinogen-coated surfaces

Adhesion of platelets to fibrinogen-coated microtiter plates was measured as previously described by Whiss and Andersson (2002). Briefly, microtiter plates (96-well MaxiSorp, Nunc, Denmark) were coated 24 h at 4 °C with 2 mg/ml of human fibrinogen (Grade L, American Diagnostica Inc., Greenwich, CT, U.S.A) in phosphate-buffered saline. Immediately before conducting the adhesion analysis, the coating solution was removed by plate inversion and gently washed with phosphate-buffered saline. Suspensions of platelets (50 μl; 1×10^8/ml) were added to the fibrinogen-coated wells at 37 °C without agitation. The samples were treated with adenosine, GEA 3175, milrinone and stimulated with thrombin for 2 min. Plate inversion and a gentle washing procedure twice in phosphate-buffered saline removed unattached platelets. The wells were then supplemented with 140 μl of 0.1 M Na-citrate/0.1 citric acid-buffer (pH 5.4), containing 1 mg/ml *p*-nitrophenyl phosphate and 0.1% Triton X-100. The plates were incubated at room temperature for 60 min with a gentle rocking motion before the reaction was stopped by adding 100 μl of NaOH (2 M). After 10 min the reaction was measured at 405 nm using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A).

The absorbance value recorded from a 50 μl cell suspension, with a density of 1×10^8/ml platelets represent 100% adhesion and the absorbance from platelet-free wells represent 0% adhesion, respectively.

2.5. Measurement of P-selectin exposure

Platelet P-selectin expression was analysed by using an enzyme-linked immunosorbent assay (Whiss et al., 1997). Aliquots (100 μl) of platelets (1×10^8/ml) were preincubated in microtiter plates with adenosine, GEA 3175 or milrinone alone and in combinations and then exposed to thrombin for 2 min. All incubations were conducted with a gentle rocking motion at 37 °C. Platelets were fixed for 5 min in phosphate-buffered saline containing 0.1% formaldehyde. After fixation, microtiter plates were centrifuged at 640 $\times g$ for 15 min, the supernatant was removed and the wells were washed with phosphate-buffered saline. Residual protein-binding sites were blocked with phosphate-buffered saline containing 5% bovine serum albumin for 1 h. The wells were washed with 0.9% NaCl containing 0.05% Tween 20. The fixed platelets were incubated with mouse monoclonal antibodies, diluted 1:2000, against P-selectin for 1 h. After another washing step with the NaCl-Tween 20 solution, antibodies bound to P-selectin were detected with secondary rabbit anti-mouse antibodies (diluted 1:1000) coupled to alkaline phosphatase. All antibodies were diluted in phosphate-buffered saline containing 1% bovine serum albumin. Following a final wash with the NaCl-Tween 20 solution, *p*-nitrophenylphosphate in dietanolamine buffer (pH 9.8) was added. Substrate hydrolysis by phosphatase was measured using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A) after 10 min incubation and data were recorded as absorbance at 405 nm.

2.6. Detection of platelet secretion of ATP

The concentration of extracellular ATP in platelet suspensions (0.5 ml; 2.5×10^8/ml) was registered using a luciferin/luciferase bioluminescent assay (Sigma Chemicals, St. Louis, MO, U.S.A). Suspensions of platelets were incubated with adenosine, forskolin, GEA 3175, milrinone alone and in combinations. Thereafter, platelet secretion was induced by adding thrombin and the ATP-dependent changes in bioluminescence were recorded in a Chronolog Dual Channel lumi-aggregometer (Model 560, Chrono-Log, Haverston, PA, U.S.A).

2.7. Measurement of fibrinogen binding

The binding of fibrinogen to platelet surface was detected by flow cytometry. The platelets (1 ml; 1×10^8/ml) were incubated with adenosine, GEA 3175 and milrinone at 37 °C in the presence of 100 μg/ml fibrinogen, followed by stimulation with thrombin for 30 s. Thereafter, the platelets were fixed with 1% paraformaldehyde for 1 h at room temperature and subjected to direct immunofluorescence staining by incubating with saturating amounts (100 μg/ml) of fluorescein isothiocyanate-conjugated polyclonal anti-human fibrinogen antibodies. The suspension of platelets and antibodies was incubated for 30 min at room temperature, washed twice in phosphate-buffered saline containing 1% bovine serum albumin and resuspended in phosphate-buffered saline. The bound fibrinogen was then analysed by flow cytometry in a Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, CA, U.S.A). The mean fluorescence values from 30,000 cells per sample were determined. Fluorescence values representing unspecific binding of the antibodies (i.e., cell suspensions with no exogenous fibrinogen and presence of EGTA) were subtracted from the values obtained from the platelet suspensions.

2.8. Measurement of cytosolic Ca^{2+}

Platelets were loaded with fura-2 by incubating platelet-rich plasma with 4 μM fura-2-acetoxymethylester (from a 4 mM stock solution dissolved in dimethyl sulfoxide) for 45 min at 20 °C. The platelets were pelleted and resuspended as described in Section 2.1. Before each measurement, 2 ml of platelet suspension ($1–2 \times 10^8$/ml) was incubated at 37 °C for 5 min and then exposed to different drugs (see Section 2.2). Fluorescence signals from platelet

suspensions were recorded using a Hitachi F-2000 fluorescence spectrofluorometer specially designed for cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, measurement. Fluorescence emission was measured at 510 nm with simultaneous excitation at 340 and 380 nm. $[Ca^{2+}]_i$ was calculated according to the general equation as described by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)(F_o/F_s)$. Maximal and minimal ratios were determined by addition of 0.1% Triton X-100 and 25 mM EGTA, respectively.

In the Mn^{2+} influx experiments, Mn^{2+} ions were added to the cells 30 s prior to thrombin. The fura-2-loaded platelets were then illuminated with 360 nm ultraviolet light and emission was detected at 510 nm; at that excitation wavelength, fura-2 fluorescence is insensitive to alteration in $[Ca^{2+}]_i$, but is quenched by the binding of Mn^{2+}. The decrease in fluorescence emission 20 s after stimulation with thrombin was set to 100%.

2.9. Determination of platelet cAMP and cGMP content

Aliquots (0.5 ml) of platelets (5×10^8/ml) were incubated with 10 μM of adenosine or GEA 3175 or both drugs. In some cell suspensions, the PDE-inhibitor milrinone (1 μM) was introduced 3 min prior to adenosine. Untreated platelet suspensions were analysed in parallel. The reaction was stopped 30 s or 30 min after introducing adenosine and GEA 3175 by adding ice-cold trichloroacetic acid (final concentration 8.3%). The suspensions were centrifuged for 10 min at 10,000 $\times g$ and supernatants were extracted with 4×2 ml of water-saturated diethylether. The aqueous phase was frozen to dryness in a vacuum freezer for 18 h and then reconstituted in Na-acetate buffer (50 mM, pH 6.2). The cGMP and cAMP levels were determined by a radioimmunoassay method using acetylated samples essentially as described by Steiner et al. (1972). Polyclonal antibodies directed towards cGMP or cAMP were prepared at our laboratory (Axelsson et al., 1988). All determinations were performed in duplicate.

2.10. Statistical methods

The results are expressed as the mean values ± standard error of the mean (S.E.M). Statistical significances were tested with one-way analysis of variance (ANOVA) with Newman–Keuls post hoc test for multiple comparisons. Statistical significance is denoted $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. The EC_{50} values were estimated from dose response curves by non-linear regression analysis of the experimental data. The EC_{50} values are shown with 95% confidence limits. The data were analysed using GraphPad Prism™ 4.0 (GraphPad Software, San Diego, CA, U.S.A).

2.11. Drugs and antibodies

GEA 3175, 1,2,3,4-Oxatriazolium, 3-(3-chloro-2-methylphenyl)-5-[[(4-methylphenyl)sulfonyl]amino]-,hydroxide inner salt (GEA Pharmaceutical Co, Copenhagen, Denmark). Adenosine and guanosine 3′:5′-cyclic phosphoric acid, 2′-O-succinyl [^{125}I]iodotyrosine methyl ester (Du Pont, Belgium); Adenosine, apyrase, acetylsalicylic acid, forskolin, fura-2-acetoxymethylester, milrinone, ODQ, and thrombin (Sigma Chemical, St. Louis, MO, U.S.A.). Human fibrinogen (Grade L, American Diagnostica Inc., Greenwich, CT, U.S.A).

The mouse monoclonal antibody against P-selectin (anti-GMP-140, IgG1 isotype, clone AC1.2) was from Becton Dickinson (San Jose, CA, U.S.A). The alkaline phosphate conjugated rabbit anti-mouse antibody (clone D314) and the fluorescein isothiocyanate-conjugated rabbit anti-human fibrinogen antibody were from Dako A/S (Glostrup, Denmark).

3. Results

3.1. Increases in platelet cyclic nucleotide content

The first objective in this investigation was to clarify the short (30 s) and long-time (30 min) action of adenosine combined with either GEA 3175 or the class 3 PDE inhibitor milrinone on platelet cAMP content. By using radioimmunoassay, we found that adenosine (10 μM; 30 s or 30 min exposure time) did not significantly influence on platelet cAMP levels (Table 1). However, adenosine added together with GEA 3175 (10 μM) or milrinone (1 μM) induced a significant and prolonged increase in cAMP. In fact, the magnitude of the cAMP response was identical when platelets were exposed to either of these two combinations of drugs. As summarised in Table 1, mono-treatment with milrinone or GEA 3175 did not affect platelet cAMP levels.

The platelet content of cGMP was markedly increased by GEA 3175 (Table 2). The magnitude of the GEA 3175-induced cGMP response was similar in the absence and presence of adenosine. Treatment with adenosine, milrinone or the combination of the two agents did not affect the cGMP levels. Control experiments revealed that the soluble guanylyl cyclase inhibitor ODQ completely antagonised GEA 3175-induced elevation of cGMP. In these experiments, suspension of platelets were exposed to 2 μM of ODQ for 5 min followed by GEA 3175 (1 μM for 2 min). The GEA 3175-stimulated cGMP levels were 0.84 ± 0.23 and 0.09 ± 0.02 pmol per 10^9 platelets in the absence and presence of ODQ, respectively. The results represent mean ± S.E.M, $n = 3$. Incubation with ODQ alone did not affect basal cGMP levels (data not shown).

3.2. Inhibition of platelet functional responses

The anti-adhesive and anti-aggregatory actions of simultaneous treatment with adenosine/GEA 3175 or adenosine/milrinone were thoroughly characterised and compared by using aggregometry, flow cytometry, and a platelet adhesion assay. Measurement of increases in light transmission through a suspension of platelets is a

Table 1
Platelet cAMP content

	Drug exposure time	
	30 s	30 min
	pmol cAMP/10^9 platelets, ($n = 6$)	
Control	6.3 ± 0.3	6.2 ± 0.5
Adenosine (10 μM)	8.1 ± 0.7	6.9 ± 0.5
GEA 3175 (10 μM)	7.6 ± 0.8	6.3 ± 0.3
Milrinone (1 μM)	7.1 ± 0.5	7.2 ± 0.3
Adenosine + GEA 3175	11.8 ± 1.1[a,b]	10.8 ± 0.6[c]
Adenosine + milrinone	12.1 ± 1.0[a,b]	10.3 ± 0.6[c]

The values are presented as mean ± S.E.M.
[a] $P<0.001$ versus control (untreated platelets) or milrinone alone.
[b] $P<0.01$ versus GEA 3175 or adenosine alone.
[c] $P<0.001$ versus control or mono-treatment with adenosine, GEA 3175, and milrinone.

Table 2
Platelet cGMP content

	Drug exposure time	
	30 s	30 min
	pmol cGMP/10^{10} platelets, (n=6)	
Control	0.5±0.0	0.5±0.1
Adenosine (10 μM)	0.5±0.1	0.5±0.0
GEA 3175 (10 μM)	44.5±6.4[a]	16.9±1.8[a]
Milrinone (1 μM)	0.4±0.0	0.5±0.0
Adenosine+GEA 3175	50.1±11.8[a]	15.4±0.1[a]
Adenosine+milrinone	0.5±0.1	0.5±0.0

The values are presented as mean±S.E.M.
[a] $P<0.001$ versus control (untreated platelets), adenosine, milrinone, or the combination of adenosine and milrinone.

classical technique for studying platelet aggregation in vitro. Fig. 1 shows the effect of adenosine (10 μM) combined with milrinone (1 μM) or GEA 3175 (10 μM) on thrombin (0.1 U/ml)-induced aggregation. Exposure of platelets to milrinone followed by adenosine delayed the onset of aggregation and decreased the aggregation speed (Fig. 1). On the other hand, the traces in Fig. 1 show that this combination only marginally influenced on the maximal aggregatory response (90.9±3.9% of thrombin-stimulated platelet aggregation, mean±S.E.M, n=6). It is to be noted that milrinone alone did not affect thrombin-induced platelet aggregation (data not shown). In comparison, adenosine administered together with GEA 3175 provoked a powerful inhibition of thrombin-induced platelet aggregation (21.6±4.9% of thrombin-stimulated platelet aggregation, mean±S.E.M, n=16). Control experiments revealed that forskolin (10 nM), which directly activates adenylyl cyclase, did not suppress platelet aggregation (97.4±2.2% of thrombin-stimulated aggregation, mean±S.E.M, n=3). Moreover, treatment with milrinone did not augment the action of forskolin (98.3±2.6% of thrombin-stimulated aggregation, mean±S.E.M, n=3). However, in common with results obtained by using adenosine, we found that GEA 3175 (10 μM) markedly enhanced the effect of forskolin. In fact, forskolin combined with GEA 3175 abolished thrombin-stimulated platelet aggregation (n=3).

The effects of adenosine (10 μM), GEA 3175 (10 μM) and milrinone (1 μM) were further evaluated by studying platelet adhesion to fibrinogen-coated surfaces (Fig. 2A). We found that the

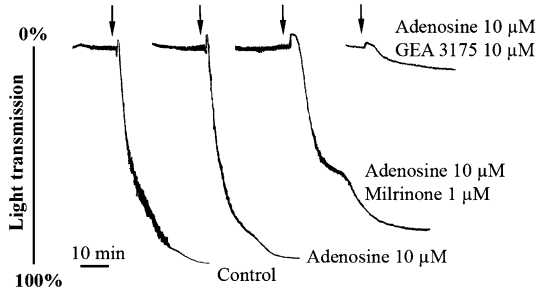

Fig. 1. Inhibition of thrombin-stimulated platelet aggregation. Isolated human platelets were incubated with combinations of adenosine (10 μM; 2 min), and GEA 3175 (10 μM; 2 min) or adenosine and milrinone (1 μM; 5 min). Thereafter the platelets were activated by adding 0.1 U/ml of thrombin (indicated by the arrows). The traces are representative for at least 6 similar experiments.

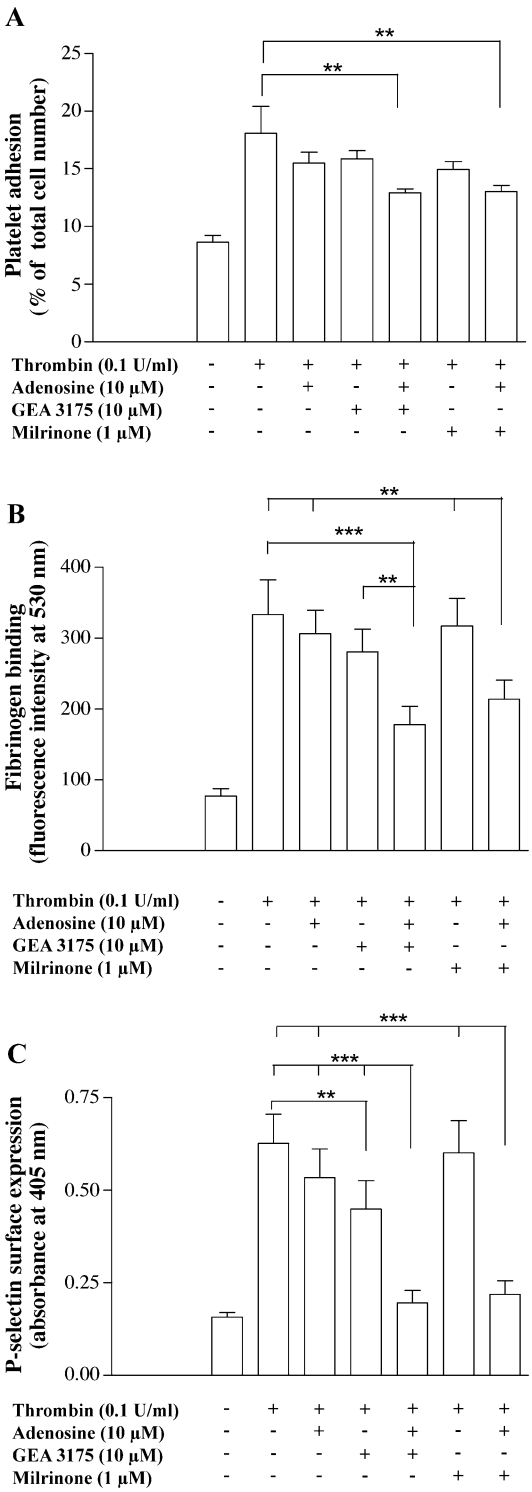

Fig. 2. Summarised effects on platelet functions. Suspensions of platelets were incubated with adenosine (10 μM), GEA 3175 (10 μM), milrinone (1 μM), alone and in combinations for 2 min (adenosine and GEA 3175) or 5 min (milrinone). The platelets were then stimulated with thrombin (0.1 U/ml) and analysed for adhesion to a fibrinogen-coated surface (A), fibrinogen binding to the surface (B), and P-selectin expression (C) as described in the material and method section. The values are expressed as mean±S.E.M, n=6. (**$P<0.01$, and ***$P<0.001$).

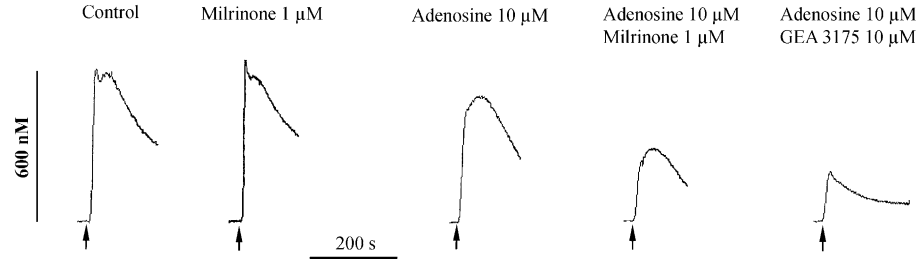

Fig. 3. Reduction of thrombin-induced rises in $[Ca^{2+}]_i$. The traces show thrombin (0.1 U/ml)-stimulated rises in $[Ca^{2+}]_i$ in fura-2-loaded platelets. Suspensions of platelets were incubated adenosine (10 μM; 2 min) combined with GEA 3175 (10 μM; 2 min) or milrinone (1 μM; 5 min) before the addition of thrombin (indicated by arrows). The effect of mono-treatment of the drugs was analysed in parallel. The traces show one of at least 8 different experiments.

substances did not influence on the adhesion of non-activated platelets to immobilised fibrinogen (data not shown). On the other hand, the increased binding of thrombin (0.1 U/ml)-stimulated platelets to the surface was markedly reduced by the different drug combinations. More specifically, simultaneous treatment with adenosine and GEA 3175 or adenosine and milrinone significantly suppressed platelet adhesion. As shown in Fig. 2A, the two combinations of drugs were equally effective in inhibiting platelet adhesion. Separately, adenosine, GEA 3175, and milrinone did not effect platelet adhesion to immobilised fibrinogen.

Flow cytometry was used to analyse directly the binding of soluble fibrinogen to platelet membrane. In accordance with the recordings of platelet aggregation and adhesion, the results revealed that only when adenosine (10 μM) was combined with GEA 3175 (10 μM) or milrinone (1 μM) did the drug significantly reduced the binding of fibrinogen (Fig. 2B). Mono-treatment with adenosine, GEA 3175, or milrinone did not significantly affect the binding of fibrinogen to thrombin-activated platelets.

Platelet secretion was analysed by measuring the upregulation of P-selectin from platelet α-granules and the release of ATP from the dense granules. The influence of adenosine (10 μM), GEA 3175 (10 μM), and milrinone (1 μM), alone and in combination, on thrombin (0.1 U/ml)-stimulated surface exposure of P-selectin is illustrated in Fig. 2C. The results show that adenosine given with GEA 3175 as well as adenosine combined with milrinone abolished the upregulation of P-selectin. Mono-treatment with adenosine and milrinone did not significantly reduce the surface expression of the cell-adhesion molecule. It is, however, to be noted that GEA 3175 alone produced a significant reduction of thrombin-stimulated surface expression of P-selectin.

By using the luciferin/luciferase bioluminescent reagent we compared the efficacy of the adenosine/GEA 3175-and the adenosine/milrinone-combination in inhibiting the release of ATP. The amount of ATP released after thrombin (0.1 U/ml)-stimulation was found to be 1.03±0.19 nmol (mean±S.E.M, $n=7$). The results revealed that adenosine combined with milrinone potently suppressed the secretion of ATP (0.24±0.05 nmol ATP, mean±S.E.M, $n=5$). On the other hand, milrinone alone did not affect thrombin-stimulated platelet secretion (data not shown). In comparison, the combination of adenosine and GEA 3175 was more effective in inhibiting ATP release. More specifically, adenosine combined with GEA 3175 abolished thrombin-stimulated ATP release in all tested platelet preparations ($n=18$). Control experiments revealed that forskolin (10 nM) decreased the liberation of ATP from platelets (0.76±0.05 nmol ATP, mean±S.E.M, $n=3$). The inhibition produced by forskolin was enhanced in milrinone-treated suspensions (0.49±0.05 nmol ATP, mean±S.E.M, $n=3$). Furthermore, forskolin combined with GEA 3175 completely blocked thrombin-stimulated liberation of ATP ($n=3$).

3.3. Effects on cytosolic Ca^{2+} responses

Rises in cytosolic Ca^{2+} were measured in fura-loaded platelet. As shown in Fig. 3, thrombin provoked a dose-dependent (see "Materials and Methods", Section 2.2) and rapid increase in $[Ca^{2+}]_i$ in platelets. The thrombin (0.1 U/ml)-stimulated peak rise in $[Ca^{2+}]_i$ in platelets was 623.7±34.4 nM (mean±S.E.M, $n=17$). The thrombin-stimulated rise in $[Ca^{2+}]_i$ was substantially reduced by adenosine (10 μM) given together with 1 μM of milrinone (initial rise: 126.6±10.0 nM, mean±S.E.M, $n=8$). The magnitude of inhibition was similar to that produced by a combination of adenosine and GEA 3175 (initial rise: 127.0±14.3 nM, mean±S.E.M, $n=12$). Thus, the results indicate that the two combinations of drugs were equally effective in suppressing peak rises in $[Ca^{2+}]_i$ in platelets. However, the traces in Fig. 3 also indicate that adenosine combined with GEA 3175 more rapidly reversed the Ca^{2+} signal to basal levels. More prolonged Ca^{2+} signals are often attributed to influx of Ca^{2+} across the plasma membrane. Therefore, we used the Mn^{2+}-quenching technique to evaluate the effects of the drugs on divalent ion influx in platelets. Fura-2-loaded platelets, in buffer supplemented with 100 μM Mn^{2+}, were stimulated with thrombin (0.1 U/ml). The effects of adenosine (10 μM), GEA 3175 (10 μM) and milrinone (1 μM) alone and in combinations on Mn^{2+}-dependent quenching of fura-2 fluorescence are summarised in Table 3. The results show the combination of adenosine and GEA 3175 induced a much more powerful inhibition of Mn^{2+} entry than adenosine combined with milrinone.

Table 3
Mn^{2+}-induced quenching of fura-2 fluorescence

	Quenching (% of control), ($n=6$)
Control	100
Adenosine (10 μM)	90.0±2.6
GEA 3175 (10 μM)	56.2±1.4[a]
Milrinone (1 μM)	87.7±3.3
Adenosine+GEA 3175	29.2±2.2[b]
Adenosine+milrinone	63.1±2.5[a]

The values are presented as mean±S.E.M.
[a] $P<0.001$ versus mono-treatment with adenosine or milrinone.
[b] $P<0.001$ versus mono-treatment with GEA 3175, milrinone, and adenosine, or adenosine combined with milrinone.

4. Discussion

It has been known for several decades that adenosine inhibits platelet functions (Haslam and Rosson, 1975). More recent studies have revealed that adenosine binds to G-protein-coupled receptors designated adenosine A_1-, A_{2A}-, A_{2B}-, and A_3-receptors (Ralevic and Burnstock, 1998). In platelets, adenosine interacts with adenosine A_{2A}-receptors and the following signal transduction pathway leads to rises in intracellular cAMP levels (Feoktistov et al., 1992; Varani et al., 1996). Elevation of this cyclic nucleotide is associated with a great number of intracellular changes (e.g., phosphorylation cascades) that ultimately results in inhibition of platelet adhesion, secretion and aggregation. In fact, cAMP-elevating prostaglandins are considered to be the most effective endogenous inhibitors of platelets (Siess et al., 1993). Despite these facts, the results in the present investigation characterise adenosine as a very weak inhibitor of thrombin-stimulated platelet responses. More specifically, we found that adenosine did not affect thrombin-induced platelet adhesion to immobilised fibrinogen, binding of fibrinogen to the membrane, and surface expression of P-selectin. One reason for this is certainly the small and insignificant effect of adenosine on platelet intracellular cAMP content. It is also to be remembered that thrombin is the most powerful activator of human platelets. This implies that thrombin, at sufficient concentrations, easily overcomes the inhibitory actions of adenosine.

In the present study, we found that adenosine added together with the PDE3 inhibitor milrinone induced a significant increase in the cAMP level in platelets. This observation shows that adenosine can produce a detectable increase in cAMP when the degradation of the cyclic nucleotide is prevented. In accordance with our findings, it has been shown that milrinone markedly enhances the actions of adenosine (Anfossi et al., 1996). Thus, our results further highlight on the importance of class 3 PDE activity in regulating platelet responsiveness. The concentration and the characteristics of specific cyclic nucleotide-elevating agents also control changes in platelet cyclic nucleotide levels. For instance, many NO-donors are unstable, short-acting drugs that produce transient rises in cGMP levels. Consequently, a drug like milrinone may induce a much more long-lasting inhibition of PDEs compared to a NO-containing compound. However, in comparison with the adenosine/milrinone combination, the results revealed that adenosine combined with the NO-containing oxatriazole derivative GEA 3175 induced a similar cAMP response. Interestingly, the increase in cAMP provoked by adenosine combined with GEA 3175 persisted over a period of 30 min. This implies that GEA 3175 mediates a long-lasting enhancement of the effect of adenosine. Indeed, GEA 3175 has been characterised as a long-acting cGMP-elevating compound in platelets and tracheal smooth muscle cells (Johansson Rydberg et al., 1997; Asplund Persson et al., 2004). Taken together, the results show that the oxatriazole derivative GEA 3175 provoked a rapid and sustained enhancement of adenosine-stimulated increases in cAMP. This effect is, most likely, attributed to GEA 3175-induced elevation of cGMP and the subsequent inhibition of PDE3.

Considering platelet functional responses, we found that adenosine combined with either GEA 3175 or milrinone significantly affected platelet degranulation. The cell adhesion molecule P-selectin is localised within the membrane of platelet α-granules. For that reason, extracellular P-selectin is upregulated as a consequence of degranulation. Our results show that both combinations of drugs abolished thrombin-stimulated surface expression of P-selectin. These findings suggest that inhibition of PDE3, by cGMP or drugs like milrinone, is a prerequisite for adenosine-induced inhibition of α-granule secretion. By mediating adhesion to leukocytes, P-selectin is important for the pro-inflammatory capacity of platelets (Furie et al., 2001). Consequently, the results suggest that inhibition of PDE3 has an anti-inflammatory effect. We also found that adenosine given with GEA 3175 was far more effective in inhibiting thrombin-stimulated release of dense granule-stored ATP. Depending on the concentration and type of activator used, the platelet granules release their content in the following order: α-granule first, thereafter the dense granules and lastly the lysosomes. In a general perspective, the results indicate that the efficacy of platelet inhibitors is also of importance in this regard. Taken together, the results show that concomitant exposure of platelets to adenosine and a NO-containing compound is necessary to achieve a complete inhibition of both α- and dense granule secretion. On the other hand, inhibition of PDE3 followed by addition of adenosine results in a potent, but incomplete suppression of platelet secretion. In addition, forskolin did not abolish thrombin-stimulated ATP release. Therefore, we suggest that activation of both the cAMP and the cGMP signal transduction pathway is a prerequisite for total inhibition of platelet secretion.

Thrombin-induced platelet aggregation was markedly reduced in cell suspensions treated with adenosine together with GEA 3175. Glycoprotein (GP) IIb/IIIa plays a pivotal role in the formation of platelet aggregates. Upon platelet activation, this integrin changes conformation and recognises arg:gly:asp-sequences on fibrinogen and other proteins. The fibrinogen molecule has two-fold symmetry, thus, it can act as a protein bridge between two adjacent platelets. Flow cytometric analysis confirmed that the combination of adenosine and GEA 3175 reduced fibrinogen binding to the platelet surface. Consequently, the results propose that the adenosine/GEA 3175-combination inhibited the conformational change of GP IIb/IIIa to its high affinity, arg:gly:asp-binding state. In comparison with results obtained by aggregometry, co-treatment with adenosine and GEA 3175 was less effective in reducing platelet adhesion to immobilised fibrinogen. A possible explanation is that fibrinogen coated on surfaces could interact with GP IIb/IIIa inde-

pendently of arg:gly:asp-sequences and without prior platelet activation (Charo et al., 1994). The combination of adenosine and the PDE3-inhibitor milrinone decreased the binding of fibrinogen to platelets, diminished platelet adhesion to immobilised fibrinogen, delayed the onset and reduced the speed of aggregation. Opposite to the effect registered by using the adenosine/GEA 3175-combination, the maximal aggregatory response was almost unaffected by co-treatment with adenosine and milrinone. Similar results were registered by replacing adenosine with forskolin. There is a large body of evidence that elevation of platelet cAMP content is associated with inhibition of platelet aggregation. However, our results clearly illustrate the importance of simultaneous activation of both the cAMP and the cGMP signal transduction pathways to achieve a more powerful inhibition of platelet aggregation. The allosteric, cGMP-mediated, inhibition of PDE3 activity is certainly one important molecular mechanism responsible for synergistic interaction between adenosine and GEA 3175. In addition, several published papers have shown that inhibitors of PDE5 (the isoenzyme that hydrolyses cGMP) enhance the action of NO-donors (Bult et al., 1991; Dunkern and Hatzelman, 2005). Consequently, the activity of PDE3 and PDE5 plays a pivotal role in platelet sensitivity towards NO-donors and cAMP-elevating drugs. However, besides modulation of PDE3 activity, the present results strongly indicate that other cGMP-specific mechanisms contribute to enhanced inhibition of platelet aggregation and secretion.

Activator-induced rises in $[Ca^{2+}]_i$ is a key event underlying the outcome of platelet functional responses. The drug combinations containing adenosine/milrinone and adenosine/GEA 3175, respectively, were equally effective in inhibiting the peak rise in $[Ca^{2+}]_i$ triggered by thrombin. This observation implies that the early phase of thrombin-triggered rises in $[Ca^{2+}]_i$ is highly sensitive to increases in cAMP. On the other hand, more prolonged Ca^{2+} responses, attributed mainly to entry of Ca^{2+}, were less suppressed by adenosine given together with milrinone. This finding indicates that inhibition of influx of Ca^{2+} represents one additional molecular mechanism, besides PDE3 inhibition, that participates in the cross-talk between adenosine and GEA 3175. More specifically, we suggest that some aspects of thrombin-induced Ca^{2+} entry are highly sensitive to the GEA 3175-induced NO/cGMP pathway. Alternatively, some Ca^{2+} influx mechanism(s) are suppressed only when cAMP and cGMP are elevated simultaneously.

There are of course other possible mechanisms by which cyclic nucleotides could interact in platelets. For example, the cytoskeleton-binding protein VASP is serine-phosphorylated by both PKA and PKG (Aszodi et al., 1999). Other possible target proteins for cyclic nucleotide-dependent kinases include tyrosine kinases and low molecular weight G-proteins. In this regard, concurrent elevation of cAMP and cGMP results in a synergistic phosphorylation of rap 1b (Reep and Lapetina, 1996). We do not exclude roles for these molecular mechanisms in the synergistic inhibition of platelet functions.

In conclusion, the presented results confirm the importance of cGMP-mediated inhibition of class 3 PDEs, resulting in enhanced efficacy of adenosine. However, the results also imply that other GEA 3175-induced cGMP-specific actions contribute to the pronounced inhibition of platelet functions. Based on the presented results we suggest that an effective reduction of Ca^{2+} entry across the plasma membrane represent another intracellular mechanism involved in the interplay between adenosine and GEA 3175. From a general point of view, these findings put emphasis on the importance of activating different signal transduction pathways in order to completely prevent platelet activation.

Acknowledgements

This work was supported by grants from Östergötlands Läns Landsting (2004/048 and 2004/050), the Swedish Heart and Lung Foundation (20030578), the Swedish Research Council (12668) and the Cardiovascular Inflammation Research Centre at Linköping University.

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