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Synergistic inhibition of thrombin-induced platelet aggregation by the novel nitric oxide-donor GEA 3175 and adenosine

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1 The influence of the novel nitric oxide-donor GEA 3175 on thrombin- and ionomycin-stimulated human platelets was investigated. The effect of GEA 3175 was compared with that of adenosine, an activator of platelet adenylyl cyclase.

2 GEA 3175 inhibited thrombin-induced secretion of ATP but did not affect aggregation; similar results were obtained with adenosine.

3 Thrombin-stimulated rises in the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, were dose-dependently inhibited by GEA 3175 and adenosine. GEA 3175 and adenosine maximally reduced the initial rise in $[Ca^{2+}]_i$ by 41% and 35%, respectively.

4 Simultaneous exposure to GEA 3175 and adenosine nearly abolished both the functional responses (i.e. aggregation and degranulation) and the rises in $[Ca^{2+}]_i$ in thrombin-stimulated platelets.

5 Aggregation and increases in $[Ca^{2+}]_i$ triggered in platelets by the Ca^{2+}-ionophore ionomycin were only marginally affected by a combination of GEA 3175 and adenosine.

6 GEA 3175 potently increased the guanosine 3':5'-cyclic monophosphate (cyclic GMP) content in platelets but did not affect adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels. Adenosine did not increase either the cyclic AMP or the cyclic GMP levels in platelets. However, adenosine and GEA 3175 combined significantly elevated the platelet cyclic AMP content.

7 The results show that simultaneous exposure to GEA 3175 and adenosine promotes potent anti-aggregatory properties in platelets *in vitro*. The findings suggest that blockage of the cytosolic Ca^{2+}-signal, which is probably mediated by an amplified cyclic nucleotide response, is an important event during the synergistic inhibition of thrombin-induced aggregation.

Keywords: Adenosine; platelets; nitric oxide; calcium; GEA 3175

Introduction

Thrombin is a powerful platelet agonist that stimulates various functional responses, like shape-change, degranulation and aggregation. The interaction between thrombin and its receptor is accompanied by several intracellular alterations in the platelet, e.g. rapid rises in the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$. This receptor-mediated increase in $[Ca^{2+}]_i$ involves influx of Ca^{2+} across the plasma membrane and liberation of Ca^{2+} from intracellular stores (Pollock & Rink, 1986; Sage *et al.*, 1989).

It has been established that elevated cyclic nucleotide levels are coupled to inhibition of platelet functions. Nitric oxide (NO) increases the guanosine 3':5'-cyclic monophosphate (cyclic GMP) content in platelets by binding to the heme-group of soluble guanylyl cyclase, which leads to activation of the enzyme (Ignarro, 1990). Adenosine inhibits agonist-stimulated platelets, and this effect is believed to be mediated by a receptor-operated activation of adenylyl cyclase and a subsequent increase in adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Haslam & Rosson, 1975; Feoktistov *et al.*, 1992; Agarwal *et al.*, 1994). Both cyclic GMP and cyclic AMP inhibit agonist-induced increases in $[Ca^{2+}]_i$ in platelets (Geiger *et al.*, 1994), and this effect is assumed to be a major target of action for cyclic nucleotides. However, the exact mechanisms underlying cyclic nucleotide-induced inhibition of platelet functions are still not completely understood. Furthermore, a combination of different compounds that increase either cyclic AMP or cyclic GMP has been shown to affect platelets in a synergistic manner (Maurice & Haslam 1990; Fisch *et al.*, 1995; Grunberg *et al.*, 1995). This indicates that intracellular cross-talk between the pathways responsible for increasing the levels of cyclic nucleotides plays an important role in the inhibition of platelet functions.

GEA 3175 is a 4-aryl-substituted oxatriazol derivative (Figure 1) that exerts biological effects by releasing NO (Kankaanranta *et al.*, 1996). GEA 3175 and a number of closely related substances have recently been shown to have a more potent effect than other, more commonly used NO-donors on a variety of cell types and tissues (Moilanen *et al.*, 1993; Corell *et al.*, 1994). In the present study, we examined the effect of GEA 3175 on the functional responses and the cytosolic Ca^{2+}-signalling in human platelets stimulated with thrombin or ionomycin. The effect of GEA 3175 was compared with that of adenosine, and possible synergistic interactions between these compounds were investigated.

Methods

Platelet preparation

Human blood was collected from healthy volunteers and immediately mixed with an acid-citrate-dextrose solution (6:1, vol:vol) composed of 85 mM Na-citrate, 71 mM citric acid and 111 mM glucose. The blood was centrifuged for 20 min at 220 *g* to obtain platelet-rich plasma (PRP). Acetylsalicylic acid (100 μM) and apyrase (1 unit ml^{-1}) were added to the PRP to prevent activation of the platelets by eicosanoids and adenine nucleotides during the preparation procedure. The PRP was then centrifuged for 20 min at 480 *g* and the supernatant was removed. The platelets were gently resuspended in a Ca^{2+}-free Tyrode-HEPES solution (pH 7.4) composed of (mM): NaCl 120, KCl 5, $MgCl_2$ 1, $NaHCO_3$ 10, HEPES 10, glucose 10 and apyrase (1 u ml^{-1}). All platelet suspensions were stored in

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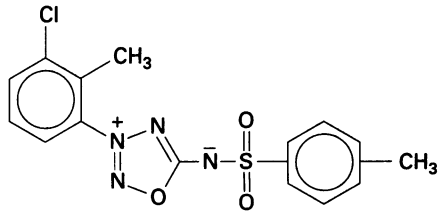

Figure 1 The chemical structure of GEA 3175.

plastic tubes at room temperature and were used within 3 h of preparation. The external Ca^{2+} concentration was adjusted to 1 mM with $CaCl_2$ immediately before each measurement. Platelets were stimulated with thrombin (0.1 u ml^{-1}) or ionomycin (0.05–0.5 μM). The effects of GEA 3175 (0.01–10 μM) and adenosine (0.1–50 μM) on platelet activation were studied by adding these compounds 2 min before stimulation. GEA 3175 and ionomycin were dissolved in dimethylsulphoxide (DMSO); the final concentration of DMSO in the cell suspensions did not exceed 0.2%. The solutions of GEA 3175 were always kept in the dark.

Platelet aggregation and secretion

Changes in light transmission and secretion of ATP were recorded simultaneously by using a Chronolog Dual Channel lumi-aggregometer (Model 560, Chrono-Log., Haverston, PA). Aliquots (0.5 ml) of platelet suspensions (2.5×10^8 ml^{-1}) were preincubated at 37°C for 2 min and then stimulated with different drugs as described above. In some experiments, fibrinogen (10 μg ml^{-1}) was added 3 min before stimulation with thrombin. The degree of aggregation triggered by thrombin or ionomycin was defined as the change in light transmission during a 10 min recording. The concentration of extracellular ATP was determined by using a luciferine/luciferase bioluminescent assay (Chrono-Log., Haverston, PA).

Measurement of $[Ca^{2+}]_i$

Platelets were loaded with fura-2 by incubating PRP with 4 μM fura-2-acetoxymethylester (from a 4 mM stock solution dissolved in DMSO) for 45 min at 20°C. The platelets were pelleted and resuspended as described above. Before each measurement, 2 ml of platelet suspension ($1-2 \times 10^8$ ml^{-1}) was incubated at 37°C for 2 min. Fluorescence signals from platelet suspensions were recorded on a Hitachi F-2000 fluorescence spectrofluorometer specially designed for measurement of $[Ca^{2+}]_i$. Fluorescence emission was measured at 510 nm with simultaneous excitation at 340 nm and 380 nm. $[Ca^{2+}]_i$ was calculated by using the general equation 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 adding 0.1% Triton X-100 and 25 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), respectively.

Determination of platelet cyclic nucleotide content

Aliquots (0.5 ml) of platelets (5×10^8 ml^{-1}) were incubated with 10 μM GEA 3175 and/or adenosine; unstimulated platelet

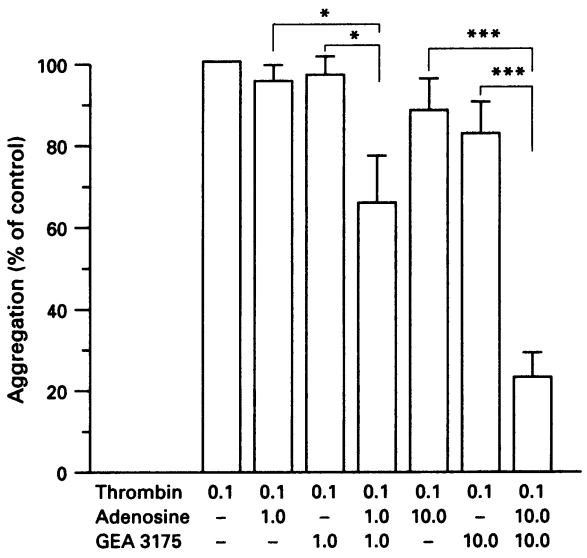

Figure 3 A summary of the effects of GEA 3175 and adenosine on thrombin-induced platelet aggregation. Platelets were incubated with GEA 3175 (1–10 μM), adenosine (1–10 μM) or a combination of both 2 min before exposure to thrombin (0.1 u ml^{-1}). Values are expressed as mean ± s.e.mean ($n = 14-18$). Statistical significance is denoted by $^*P < 0.05$ and $^{***}P < 0.001$.

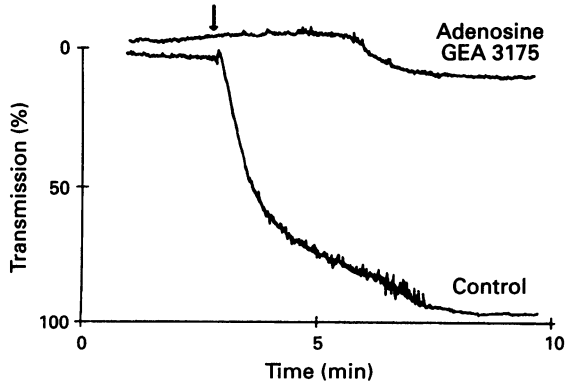

Figure 2 The effect of simultaneous exposure to GEA 3175 and adenosine on thrombin-induced platelet aggregation. Control: aggregation was induced by adding 0.1 u ml^{-1} of thrombin (arrow). The addition of GEA 3175 (10 μM) and adenosine (10 μM) 2 min before thrombin potently inhibited the aggregation. The traces are representative of 18 similar experiments.

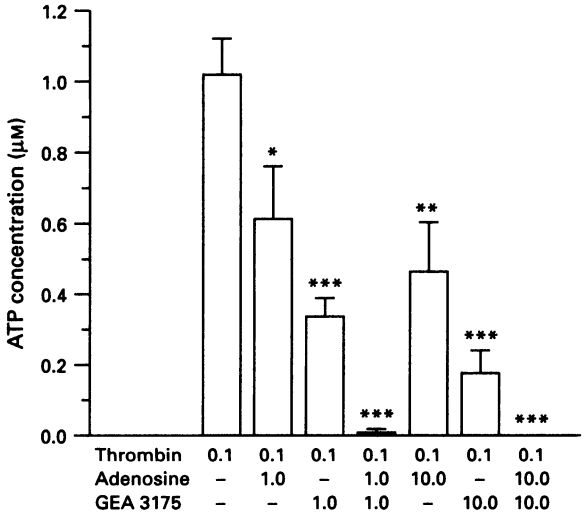

Figure 4 Inhibition of thrombin-stimulated ATP release. Aliquots of platelet suspension were preincubated with GEA 3175 (1–10 μM), adenosine (1–10 μM) or a combination of both compounds 2 min before stimulation with thrombin (0.1 u ml^{-1}). Values are expressed as mean ± s.e.mean ($n = 14-18$). Statistical significance was tested against thrombin-stimulated platelet suspensions ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

suspensions were analyzed in parallel. The reaction was stopped after 2 min by adding ice-cold trichloroacetic acid (final concentration 8.3%). Thereafter, the suspensions were centrifuged for 10 min at 10,000 *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 sodium acetate buffer (50 mM, pH 6.2). The levels of cyclic GMP and cyclic AMP were determined by a radioimmunoassay method with acetylated samples (Axelsson *et al.*, 1988). All determinations were performed in duplicate.

Statistical methods

The values are expressed as mean ± s.e.mean and statistical significance was tested by using Student's unpaired *t* test.

Drugs

GEA 3175 (1,2,3,4-oxatriazolium, 3-(3-chloro-2-methylphenyl)-5-[[(4-methylphenyl)sulphonyl]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, fibrinogen, fura-2-acetoxymethylester, ionomycin and thrombin (Sigma Chemical Co., St. Louis, MO, U.S.A.).

Results

Thrombin (0.1 u ml^{-1}) induced a potent aggregatory response in platelet suspensions, as shown in Figure 2. Neither GEA 3175 (1 – 10 μM) nor adenosine (1 – 10 μM), added 2 min before thrombin, inhibited this cellular response. However, when GEA 3175 and adenosine were added together, the aggregation was almost abolished (illustrated in Figure 2 and summarized in Figure 3). The synergistic effect of GEA 3175 and adenosine was of similar potency in the presence of 10 μg ml^{-1} fibrinogen (9.7 ± 3.4% of thrombin-induced aggregation; mean ± s.e.mean, $n = 5$). In contrast, the aggregation induced by the Ca^{2+}-ionophore ionomycin (0.5 μM) was only marginally affected by a combination of GEA 3175 (10 μM) and adenosine (10 μM) (74.0 ± 5.5% of ionomycin-induced aggregation; mean ± s.e.mean, $n = 6$).

The secretion of ATP following stimulation with thrombin was measured by using the luciferine/luciferase bioluminescent assay. Thrombin caused the liberation of μM amounts of ATP within 20 – 30 s. The thrombin-induced secretion was significantly reduced by either GEA 3175 (1 – 10 μM) or adenosine (1 – 10 μM) and was completely inhibited by coadministered GEA 3175 and adenosine (Figure 4).

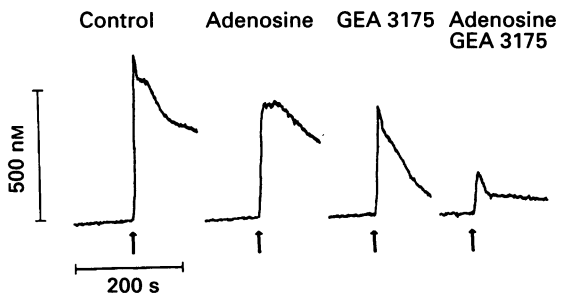

Figure 5 Typical traces obtained from fura-2-loaded platelets. Changes in $[Ca^{2+}]_i$ were induced by adding 0.1 u ml^{-1} of thrombin (indicated by arrows) in the absence (control) or presence of GEA 3175 (10 μM), adenosine (10 μM), or a combination of the two. The inhibitory compounds were added 2 min before thrombin exposure. The traces are representative of 11 similar experiments.

Typical traces from fura-loaded platelets are shown in Figure 5. The addition of thrombin provoked a potent and prolonged rise in $[Ca^{2+}]_i$ (initial rise: 629.3 ± 32.7 nM; mean ± s.e.mean, $n = 21$). GEA 3175 (0.01 – 10 μM) or adenosine (0.1 – 50 μM) dose-dependently inhibited the initial increase in $[Ca^{2+}]_i$ triggered by thrombin (Figure 6). As shown in Figure 7, the addition of both GEA 3175 and adenosine potently reduced the increase in $[Ca^{2+}]_i$ induced by thrombin. In contrast, GEA 3175 and adenosine added together were without effect on the ionomycin-induced rise in $[Ca^{2+}]_i$ (initial rise: 424.2 ± 35.0 nM and 468.8 ± 85.9 nM in the absence and presence of the adenosine-GEA 3175 combination, respectively; mean ± s.e.mean, $n = 4$, Figure 8).

The effects of GEA 3175 and adenosine on the cyclic nucleotide content in platelets are shown in Figure 9. Incubation of the cell suspensions with 10 μM adenosine for 2 min did not induce any detectable changes in the cyclic AMP or cyclic GMP content. GEA 3175 (10 μM) induced an approximately 50 fold increase in the cyclic GMP level but had no significant

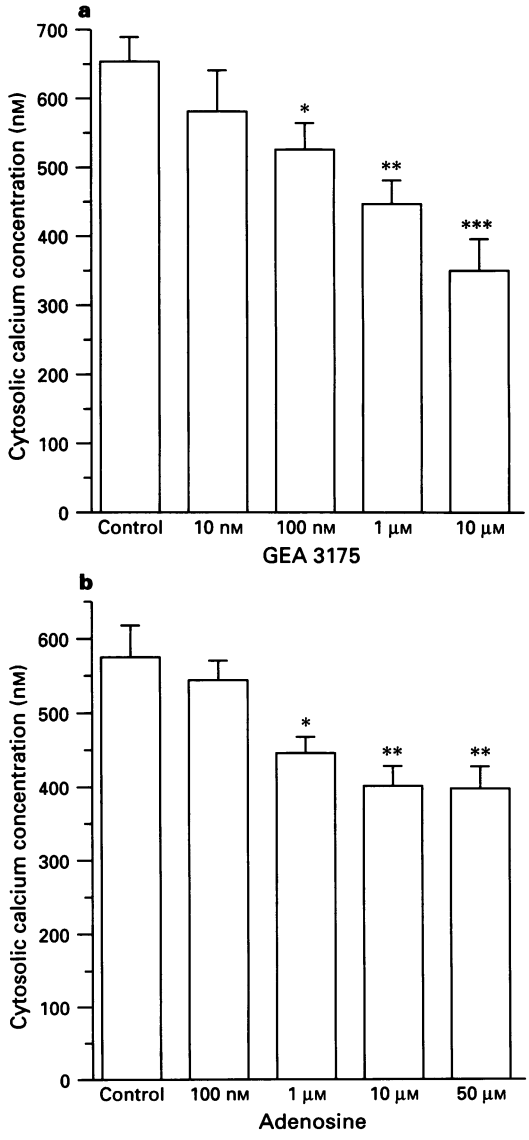

Figure 6 The dose-response effects of GEA 3175 and adenosine on the initial rise in $[Ca^{2+}]_i$ evoked by thrombin (0.1 u ml^{-1}). Fura-2-loaded platelets were incubated for 2 min with various concentrations of either GEA 3175 (a) or adenosine (b) before the addition of thrombin. Values are expressed as mean ± s.e.mean ($n = 6$). Statistical significance was tested against thrombin-stimulated platelet suspensions (control) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

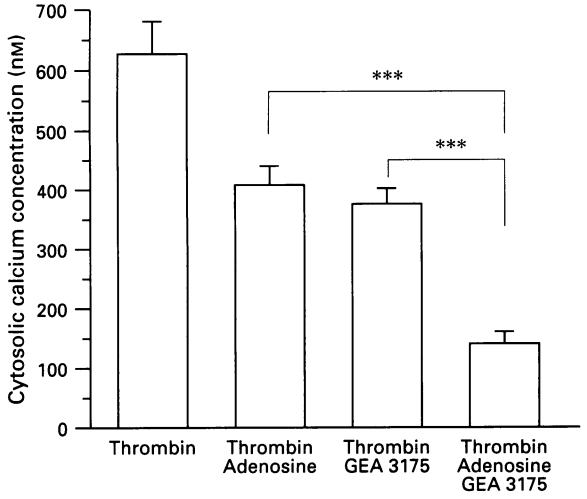

Figure 7 The effect of GEA 3175 and adenosine on the initial rise in $[Ca^{2+}]_i$ triggered by thrombin. Fura-2-loaded platelets were stimulated with thrombin ($0.1\,u\,ml^{-1}$) in the absence or presence of GEA 3175 ($10\,\mu M$) or adenosine ($10\,\mu M$) or a combination of both compounds. GEA 3175 and adenosine were introduced to the cell suspensions 2 min before stimulation with thrombin. Values are expressed as mean ± s.e.mean ($n = 11-12$). Statistical significance is denoted by $***P<0.001$.

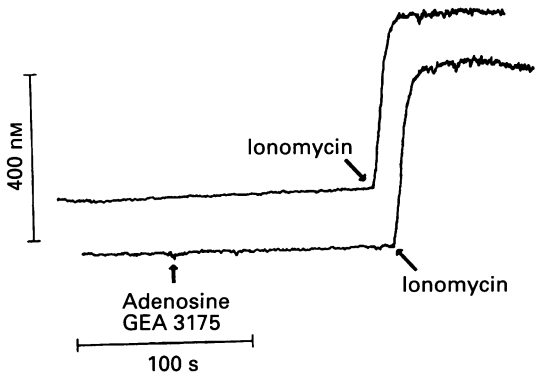

Figure 8 Changes in $[Ca^{2+}]_i$ induced by ionomycin (50 nM). Fura-2-loaded platelets were preincubated with or without a combination of GEA 3175 ($10\,\mu M$) and adenosine ($10\,\mu M$) 2 min before stimulation with ionomycin, as indicated by the arrows. The traces are representative of 4 similar experiments.

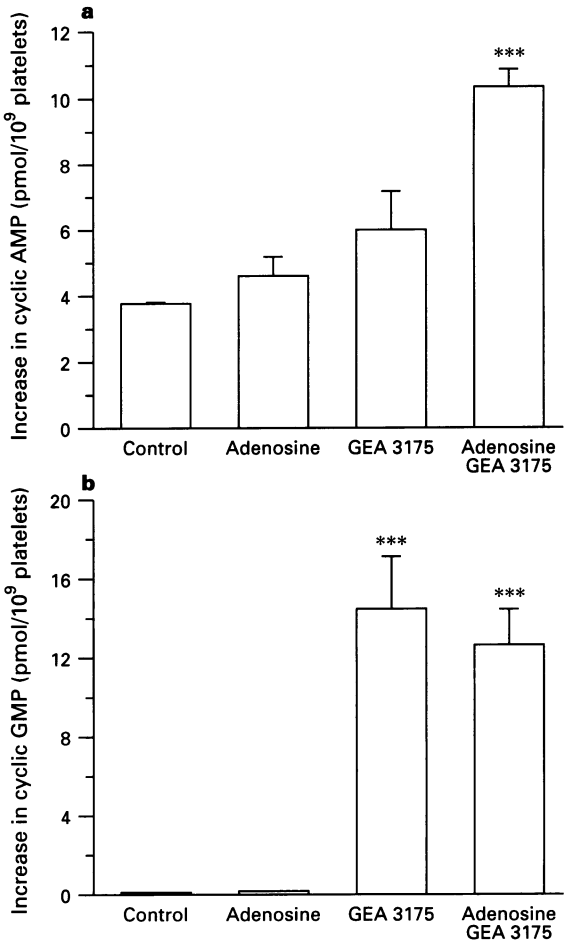

Figure 9 Increase in platelet cyclic AMP (a) and cyclic GMP (b) levels induced by GEA 3175 and adenosine. Aliquots of platelet suspensions were incubated with GEA 3175 ($10\,\mu M$) or adenosine ($10\,\mu M$) or a combination of both compounds for 2 min. Cyclic nucleotide levels were then determined by radioimmunoassay. Values are expressed as mean ± s.e.mean ($n = 6$). Statistical significance was tested against unstimulated platelet suspensions (control) ($***P<0.001$).

effect on the cyclic AMP level. However, the presence of GEA 3175 together with adenosine significantly elevated the cyclic AMP content.

Discussion

The present study shows that both the NO-donor GEA 3175 and adenosine significantly reduce secretion of ATP and rises in $[Ca^{2+}]_i$ in platelets stimulated with the potent agonist thrombin. In contrast, these inhibitory compounds did not affect thrombin-induced platelet aggregation. Other investigators have demonstrated that NO-donors inhibit platelet functions, and the effects of NO are thought to be mediated by an increase in platelet cyclic GMP content (Gerzer *et al.*, 1988; Radomski *et al.*, 1992). In support of this, our results show that GEA 3175 is indeed a potent activator of platelet guanylyl cyclase. Inhibitory effects of adenosine are believed to be mediated by an increase in platelet cyclic AMP content (Feoktistov *et al.*, 1992). In our study, a concentration of adenosine that significantly inhibited thrombin-induced degranulation and cytosolic Ca^{2+} signals did not induce any detectable increase in platelet cyclic AMP content. It is possible that adenosine induces a minor and transient increase in cyclic AMP, that rapidly returns close to the basal level. Alternatively, our findings may indicate that the effects evoked by adenosine are, at least in part, mediated by mechanisms other than an elevation of cyclic AMP. In this context, Cronstein *et al.* (1992) have proposed that adenosine inhibits chemotactic-peptide-stimulated neutrophils in a cyclic AMP-independent manner.

We found that GEA 3175 and adenosine were almost equally effective in inhibiting the initial increase in $[Ca^{2+}]_i$ triggered by thrombin. However, the reversal of the elevated Ca^{2+} levels was much more rapid in GEA 3175-treated platelets than in adenosine-treated cells (see Figure 5). Thrombin-stimulated rises in $[Ca^{2+}]_i$ are thought to involve both a rapid release of Ca^{2+} from intracellular stores and an initially rapid influx of Ca^{2+} across the plasma membrane, followed by a more prolonged influx of Ca^{2+} (Pollock & Rink, 1986; Sage *et al.*, 1989; Hashimoto *et al.*, 1992). Consequently, our findings indicate that GEA 3175, but not adenosine, inhibits the prolonged influx of Ca^{2+} across the plasma membrane.

When administered together, GEA 3175 and adenosine induced a synergistic inhibition of thrombin-stimulated platelet aggregation. A synergistic interaction between activators of cyclic GMP and cyclic AMP has previously been documented in platelets (Fisch *et al.*, 1995; Grunberg *et al.*, 1995). The

underlying mechanism is thought to involve cyclic GMP-induced inhibition of class III phosphodiesterases (Maurice & Haslam, 1990). This idea is supported by our observation that GEA 3175 and adenosine significantly elevated cyclic AMP levels only when platelets were exposed to both compounds at the same time. Our findings imply that GEA 3175 markedly increases platelet content of cyclic GMP, which subsequently enhances the weak effect of adenosine on adenylyl cyclase by inhibiting the degradation of cyclic AMP. The elevation of both cyclic GMP and cyclic AMP was associated with a potent inhibition of thrombin-stimulated rises in $[Ca^{2+}]_i$. In contrast, neither basal nor ionomycin-induced rises in $[Ca^{2+}]_i$ were affected by exposure to GEA 3175 and adenosine together. We suggest that the synergistic inhibition of thrombin-induced platelet aggregation is due to the amplified cyclic nucleotide response which effectively inhibits rises in $[Ca^{2+}]_i$. The importance of the Ca^{2+}-blockage is demonstrated by our findings that cotreatment with GEA 3175 and adenosine only marginally inhibited ionomycin-induced aggregation.

It has been shown that various functions in platelets are activated by different levels of $[Ca^{2+}]_i$. For instance, a much greater rise in $[Ca^{2+}]_i$ is required to cause agonist-induced degranulation than to activate the fibrinogen receptor on the plasma membrane (Scrutton, 1993). In our experiments, GEA 3175 or adenosine partly inhibited thrombin-induced rises in $[Ca^{2+}]_i$, which, in turn, reduced secretion but did not affect aggregation. The marked suppression of thrombin-triggered rises in $[Ca^{2+}]_i$ in platelets treated simultaneously with GEA 3175 and adenosine might prevent the conversion of the fibrinogen receptor to its active stage and thereby inhibit the aggregation. Alternatively, the lack of aggregation might be due to the abolition of the platelet secretory response. We measured only the dense-granule compound ATP, but it is likely that GEA 3175 and adenosine also blocked the secretion of substances from other platelet granules (i.e. α-granules and lysosomes). In an isolated cell suspension, reduced secretion from platelet α-granules would probably lead to insufficient release of fibrinogen and other adhesive proteins that are necessary for platelet aggregation. However, the first mechanism is relevant to the observations that GEA 3175 and adenosine together potently inhibited thrombin-induced aggregation even in the presence of externally added fibrinogen.

The present results show that, separately, GEA 3175 and adenosine are weak inhibitors of thrombin-activated platelets, whereas together the two compounds are potent blockers of platelet functional responses. This action probably involves an increased accumulation of cyclic nucleotides that effectively inhibits thrombin-stimulated rises in $[Ca^{2+}]_i$ and the subsequent aggregatory response. In conclusion, our findings indicate that a powerful anti-aggregatory therapy would require a cooperative interaction between several intracellular inhibitory pathways.

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