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Characterisation of GEA 3175 on human platelets; comparison with *S*-nitroso-*N*-acetylpenicillamine

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Abstract

By comparing the effect of two nitric oxide (NO)-containing compounds, we found that *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), but not GEA 3175 (1,2,3,4-Oxatriazolium,3-(3-chloro-2-metylphenyl)-5-[[(4-methylphenyl)sulfonyl]amino]-, hydroxide inner salt), released NO. Despite this, both drugs elevated cyclic guanosine 3′,5′-monophosphate (cGMP) levels in human platelets. However, SNAP was more effective after short exposure times (5 and 20 s). The compounds also inhibited thrombin-induced rises in cytosolic Ca^{2+}. Time studies revealed that the action of SNAP rapidly declined by increasing the length of incubation (from 5 s to 30 min). This desensibilisation phenomenon mainly involved the release of Ca^{2+} from intracellular stores. In comparison, GEA 3175-induced inhibition of cytosolic Ca^{2+} signalling was much more long-lasting. The soluble guanylyl cyclase (sGC) inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) reversed the effect of GEA 3175 on cytosolic Ca^{2+}. Consequently, this inhibition depends solely on the increase in cGMP. In summary, differences between GEA 3175 and SNAP were observed in NO releasing, cGMP elevating and Ca^{2+} suppressive properties.

Keywords: NO (Nitric Oxide); cGMP (cyclic guanosine 3′,5′-monophosphate); Calcium; Aggregation; Platelet; SNAP (*S*-nitroso-*N*-acetyl-D, L-penicillamine); GEA 3175

1. Introduction

Nitric oxide (NO) is synthesised from the vascular endothelium and acts as a potent vasodilator (Furchgott and Zawadzki, 1980; Palmer et al., 1987). Dysfunction in NO synthesis is thus considered to have a major contributory role in the development of cardiovascular diseases such as hypertension, coronary artery disease and heart failure (Megson, 2000). NO also seems to protect against thrombosis by inhibiting different platelet functions, e.g., adhesion (Radomski et al., 1987b), secretion (Grenegård et al., 1996) and aggregation (Grenegård et al., 1996; Radomski et al., 1987a). NO-containing drugs mimic the action of NO and are clinically used in the management of angina pectoris. These compounds (often referred to as organic nitroesters) are, however, less effective in inhibiting platelet responses (Martin et al., 2000). This is probably due to the incapability of platelets to metabolise the drugs, i.e., to provoke release of NO from the organic nitroester (Torfgard and Ahlner, 1994). On the other hand, there are many classes of NO-containing compounds, like *S*-nitrosothiols and sydnonimine metabolites, that in vitro effectively inhibit platelet function (Kankaanranta et al., 1996). This inhibition is presumably mediated via a NO-induced activation of soluble guanylyl cyclase (sGC) followed by an increased guanosine cyclic 3′,5′-monophosphate (cGMP) level. cGMP principally acts by regulating phosphodiesterase activity and by activating protein kinase-G (PK-G). In platelets, the 46/50 kDa vasodilator-stimulating phosphoprotein (VASP) is a main substrate protein for PK-G (Walter et al., 1993). Furthermore, one important consequence of elevated cGMP is inhibition of agonist-induced rises in $[Ca^{2+}]_i$ in platelets (Schwarz et al., 2001). The links between activation of PK-G, VASP phosphorylation and inhibition of cytosolic $[Ca^{2+}]$ responses are, however, unclear.

Mesoionic 3-aryl-substituted oxatriazole derivatives represent a poorly characterised class of NO-containing compounds with anti-platelet activity (Grenegård et al., 1996; Kankaanranta et al., 1996). In this study, we compared the

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efficacy of one of these substances, 1,2,3,4-Oxatriazolium, 3-(3-chloro-2-metylphenyl)-5-[[(4-methylphenyl)sulfonyl] amino]-, hydroxide inner salt (GEA 3175), with that of the commonly used *S*-nitrosothiol *S*-nitroso-*N*-acetyl-D, L-penicillamine (SNAP), by analysing NO release, cGMP elevation, phosphorylation of VASP and cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$. Furthermore, the anti-aggregatory capacity of GEA 3175 and SNAP in whole blood impedance measurements were investigated.

2. Materials and methods

2.1. Isolation of human platelets

Human venous blood was collected from healthy volunteers and immediately mixed with an acid-citrate–dextrose solution (6:1, v/v) 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 (PRP). Acetylsalicylic acid (100 μM) and apyrase (0.5 u 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 $480 \times g$ and the supernatant was removed. The platelets were gently resuspended in Ca^{2+}-free HEPES solution (pH 7.4) composed of 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES and apyrase (1 u ml^{-1}). The platelet suspensions were stored in plastic tubes at room temperature and were used within 3 h of preparation. Immediately before each measurement, the extracellular $[Ca^{2+}]$ was adjusted to 1 mM and the temperature was set to 37 °C.

2.2. Measurement of NO

Amperometric measurements of NO were performed with the ISO-NO Mark II NO-meter [World Precision Instruments (WPI), Sarasota, FL, USA]. The analogue signal from ISO-NOMKII meter was digitalized using a two-channel data acquisition system (DUO 18 Precision Instruments) connected to a Pentium II PC. Calibrations of the signals in terms of concentration of NO were done according to the ISO-NO Mark II instruction manual. The analysis was performed under different experimental conditions: in HEPES buffer (pH 7.4), in the presence of 10% human plasma and in platelet suspensions with a cell density of 10^7 platelets/ml. The data were recorded under constant stirring (800 rpm) and at room temperature or at 37 °C. NO release was measured from GEA 3175 and SNAP at concentrations between 0.1 and 100 μM.

2.3. Determination of platelet cGMP content

Suspensions of platelets (2.5×10^8/ml, 500 μl) were prewarmed under stirring (800 rpm) for 3 min where after different concentrations of SNAP (1 nM–10 μM) or GEA (0.1–10 μM) were added. The reaction was stopped after different periods of time (5 s up to 30 min) by adding ice-cold trichloroacetic acid (8.3% final concentration). Thereafter, the suspensions were centrifuged for 15 min at $4000 \times g$ and supernatants were extracted with 4×2 ml water-saturated diethyl ether. 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 levels of cGMP were determined by radioimmunoassay (Axelsson et al., 1988) and all determinations were performed in duplicate.

2.4. Measurement of cytosolic calcium concentration

The platelets were loaded with fura-2 by incubating PRP with 3 μM fura-2-acetoxymethylester for 45 min at 20 °C and then pelleted and resuspended in HEPES buffer as described under isolation of human platelets. Suspensions of fura-2-loaded platelet (2 ml; $1–2 \times 10^8$ cells/ml) were incubated at 37 °C for 3 min and then exposed to the NO-containing drugs SNAP (10 μM) or GEA 3175 (10 μM) for different periods of times (5 s, 2 or 30 min). In the experiments with 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), suspensions of fura-2-loaded platelet were preincubated with ODQ (2 μM) 5 min before addition of GEA 3175 (1 μM). Rises in cytosolic-free calcium concentration $[Ca^{2+}]_i$ was then induced by adding thrombin (0.1 u ml^{-1}). Fluorescence signals from platelet suspensions were recorded on a Hitachi F-2000 spectrofluorometer specially designed for measurement of $[Ca^{2+}]_i$. Fluorescence emission was registered at 510 nm during simultaneous excitation at 340 and 380 nm. $[Ca^{2+}]_i$ was calculated by using the general equation described by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)$ (Fo/Fs). Maximal and minimal ratios were determined by first adding 0.1% Triton X-100 and then 25 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA).

Fura-2-loaded platelets were also stimulated with thrombin in medium containing 0.5 mM EGTA or 100 μM Mn^{2+}. EGTA was added immediately before each measurement, and in this series of experiments, Ca^{2+} ions (4 mM) were reintroduced 3 min after thrombin stimulation.

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 this 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%.

2.5. Phosphorylation of VASP

Isolated platelets ($1–2 \times 10^9$, 100 μl) were prewarmed for 3 min in 37 °C and $[Ca^{2+}]$ was set to 1 mM. After

incubation with GEA 3175 (10 μM) or SNAP (10 μM) for 2 min, reaction was stopped by mixing Laemmli buffer [Bio-Rad, Hercules, CA, USA: composed of 62.5 mM Tris–HCl, 25% glycerol, 2% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue and 5% mercaptoethanol, pH 6.8] 1:2 and heating at 95–97 °C for 5 min. In other experiments, the platelets were incubated with GEA 3175 (10 μM) or SNAP (10 μM) at room temperature for 25 min. In the last 5 min, [Ca^{2+}] was set to 1 mM and the temperature was set to 37 °C before the reaction was stopped as described above. The samples were stored at −70 °C until used. The heating procedure was performed again before separating the aliquots on 7.5% SDS-polyacrylamide gels (SDS-PAGE) using a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad). The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerca, MA, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked for 1 h at room temperature or overnight at 4 °C with 5% (w/v) dry milk and 0.2% (v/v) Tween 20 in PBS pH 7.4 composed of 10 mM phosphate buffer and 150 mM NaCl to minimize unspecific binding. A mouse monoclonal antibody (16C2) (Alexis Biochemicals, Qbiogenic, CA, USA) to VASP at Ser^{239} was used at a dilution of 1:1000. The secondary antibody was horseradish-peroxidase-conjugated goat anti-mouse from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diluted to 1:10 000. The membranes were rinsed between incubations in 0.2% (v/v) Tween 20 in PBS and were diluted into 0.2% (v/v) Tween 20 in PBS. The membranes were detected using ECL Western blotting detection reagents (Amersham Biosciences, UK) in LAS-1000 imaging analyser (Fuji Photo Film, Tokyo, Japan).

2.6. Whole blood aggregometry

Increases in impedance were recorded by using a Chronolog Dual Channel lumiaggregometer (Model 560, Chrono-Log, Haverston, PA, USA). Heparinized whole blood (0.5 ml) was mixed with 0.9% NaCl (0.5 ml) solution and stirred at 800 rpm at 37 °C. This diluted and anticoagulant blood was incubated with SNAP (30 μM) or GEA 3175 (30 μM), and then stimulated with thrombin-receptor activated peptide (TRAP; 10 μg ml^{-1}). In another series of experiments, the platelets were activated by ADP (40 μM). Whole blood aggregation was measured as the increase in impedance between two platinum electrodes. Maximal aggregatory response to ADP and TRAP was defined as the increase in impedance after 8 min recording.

2.7. Drugs

SNAP was from ALEXIS (San Diego, CA, USA). Stock solution was kept in −70 °C. GEA 3175 was a generous gift from GEA Pharmaceutical (Copenhagen, Denmark). Stock solution of this NO-containing drug was prepared on the day of use. SNAP and GEA 3175 were dissolved in dimethylsulfoxide (DMSO); the final concentration of DMSO in the cell suspensions did not exceed 0.2% and as both these drugs are sensitive to light, the solutions were kept in foiled-wrapped vials. TRAP (SFLLRN) was a gift from Prof. N.O. Solum, Oslo, Norway.

ADP, apyrase, acetylsalicylic acid, fura-2-acetoxymethylester and thrombin were from Sigma (St. Louis, MO, USA).

2.8. Statistical analysis

Results are expressed as the mean values ± standard error of the mean (S.E.M.). Statistical significance was tested with one-way analysis of variance (ANOVA), with Newman–Keuls or Dunnett's post hoc test for multiple comparisons. Data were analysed using GraphPad Prism™ v. 4.0. (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. NO release and cGMP increase in isolated human platelets

By using the ISO-NO mark II NO-meter, we found that SNAP rapidly and dose-dependently liberated NO in HEPES buffer (Fig. 1). The release of NO from SNAP was detectable down to a concentration of 0.1 μM (data not shown). In contrast, high concentrations of the oxatriazole

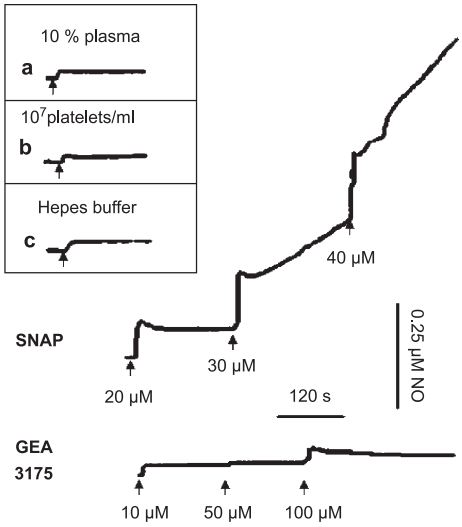

Fig. 1. Typical traces registered by the ISO-NO mark II NO-meter. These experiments were performed in 37 °C and constant stirring (800 rpm). Different concentrations of SNAP (20, 30 and 40 μM) or GEA 3175 (10, 50 and 100 μM) were added (at the arrows) to the cell-free HEPES buffer. The inserted picture shows traces from experiments in (a) and (b) with GEA 3175 (50 μM) or (c) DMSO (equal volume), added at the arrows, in 10% plasma, 10^7 platelets/ml or HEPES buffer. These experiments were performed at room temperature with constant stirring (100 rpm).

derivative GEA 3175 (10–100 μM) did not induce NO liberation. To further evaluate the capacity of GEA 3175 to release NO, experiments were performed in HEPES buffer containing plasma (10%) or isolated platelets (1×10^7/ml). However, GEA 3175 failed to induce detectable NO response even in these experimental designs (inset in Fig. 1). To clarify the cGMP elevating capacity, platelet suspensions were exposed for 2 min to 0.1–10 μM of SNAP or GEA 3175. We found that both SNAP and GEA 3175 dose-dependently increased cGMP content. As shown in Fig. 2, SNAP was found to be slightly more potent compared to GEA 3175. Additional experiments indicated that the SNAP-induced cGMP response in platelets was detectable down to a concentration of 10 nM. More specifically, 2-min exposure of, respectively, 1 and 10 nM of SNAP resulted in 0.30 ± 0.06 and 0.40 ± 0.1 pmol cGMP/10^9, $n = 5$, mean values ± S.E.M. In these experiments, the level of cGMP in untreated cell suspensions was 0.27 ± 0.04 cGMP/10^9 platelets. Time studies revealed that SNAP, compared to GEA 3175, more effectively elevated intracellular cGMP levels after short time (5 and 20 s) of incubation (Fig. 3A). After longer times (2 and 30 min) of incubation of SNAP, the cGMP levels were much lower (Fig. 3B). However, the cGMP content was still considerably higher compared to the control (untreated platelets) and no significant difference was registered between the nitrosothiol and the oxatriazole derivatives. Control experiments indicated that exposure of platelets to SNAP for 30 and 60 s resulted in lower cGMP levels compared to the 20 s incubation time (data not shown). Based on the results, we principally

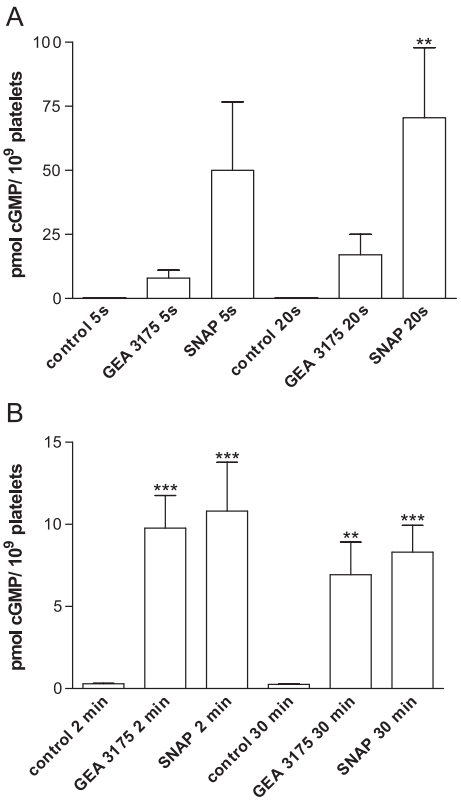

Fig. 3. Increase in platelet cGMP levels induced by SNAP (10 μM) or GEA 3175 (10 μM). Aliquots of platelet suspensions were incubated 5 and 20 s (shown in A) and 2 and 30 min (shown in B) with each drug in 37 °C. The cGMP contents were determined with radioimmunoassay as described in Determination of platelet cGMP content. Data are presented as mean values ± S.E.M. ($n = 7$). Statistical significance was tested with ANOVA followed by Dunnett's multiple comparison test as post hoc test. Significances were defined as $^{**}P < 0.01$ and $^{***}P < 0.001$.

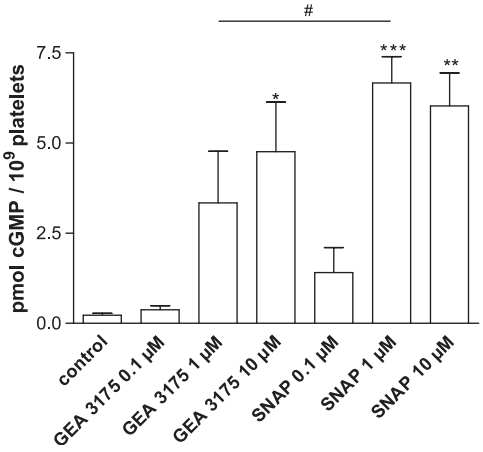

Fig. 2. Increase in platelet cGMP levels induced by GEA 3175 and SNAP. Aliquots of platelet suspensions were incubated for 2 min with 0.1, 1 or 10 μM of GEA 3175 or SNAP in 37 °C. The cGMP contents were determined with radioimmunoassay as described in Determination of platelet cGMP content. Results are presented as mean values ± S.E.M. ($n = 5$). Statistical significance was tested with ANOVA, followed by Newman–Keuls multiple comparison test as post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ against the control and between GEA 3175 (1 μM) and SNAP (1 μM) $^{\#}P < 0.05$.

conclude that maximal rise in cGMP in platelets takes place in the first 20 s after adding SNAP.

3.2. Inhibition of $[Ca^{2+}]_i$ in isolated human platelets

Exposure of fura-2-loaded platelets to SNAP (10 μM) and GEA 3175 (10 μM) resulted in suppression of thrombin

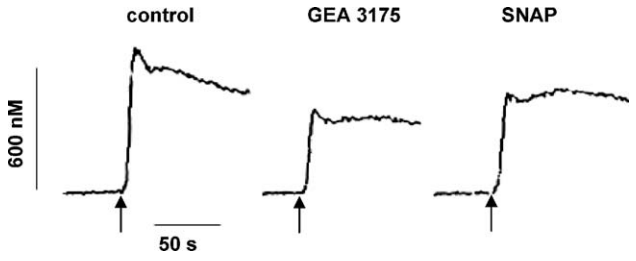

Fig. 4. Typical traces obtained from fura-2-loaded platelets. The platelets were preincubated for 2 min with SNAP (10 μM) or GEA 3175 (10 μM) and then stimulated by thrombin (0.1 u ml^{-1}) indicated by the arrows. The figure illustrates 1 of 10 similar experiments.

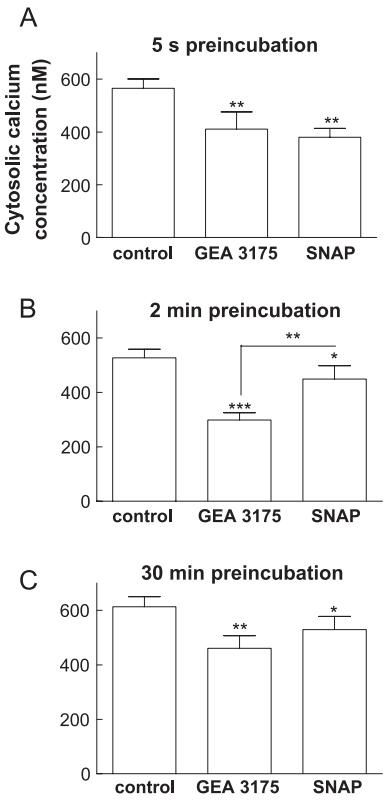

Fig. 5. The summarised effects of SNAP (10 μM) and GEA 3175 (10 μM) on the initial rise in $[Ca^{2+}]_i$ evoked by thrombin. Fura-2-loaded platelets were incubated during 5 s (A), 2 min (B) and 30 min (C) with SNAP or GEA 3175 before addition of thrombin (0.1 u ml^{-1}). Results are expressed as mean values ± S.E.M. ($n = 7–10$). Statistical significance was tested with ANOVA followed by Newman–Keuls multiple comparison test as post hoc test. *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

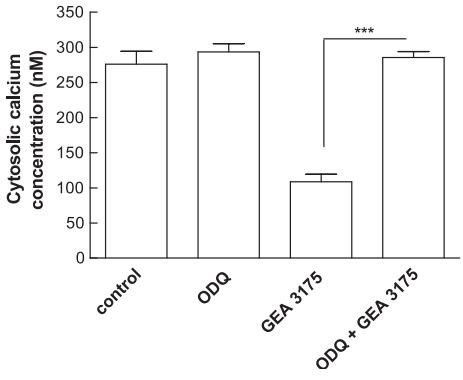

Fig. 6. The summarised effect of GEA 3175 (1 μM) and ODQ (2 μM) and the combination of the two drugs on the initial rise in $[Ca^{2+}]_i$ evoked by thrombin. Fura-2-loaded platelets were preincubated during 5 min with ODQ before addition of GEA 3175 and, after further 5 min, stimulated with thrombin (0.1 u ml^{-1}). Results are expressed as mean values ± S.E.M. ($n = 5$). Statistical significance was tested with ANOVA followed by Newman–Keuls multiple comparison test as post hoc test. *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

(0.1 u ml^{-1})-induced rises in $[Ca^{2+}]_i$ (Fig. 4). Fig. 5 shows that the effect of SNAP was most pronounced after short time of incubation (5 s). After longer periods of incubation (2 and 30 min), the Ca^{2+}-suppressive action of SNAP markedly declined. In comparison, the Ca^{2+}-lowering capacity of GEA 3175 was more long-lasting (Fig. 5). It is noteworthy that thrombin-induced rises in $[Ca^{2+}]_i$ were almost identical after 5 s up to 30 min of preincubation of DMSO (see Fig. 5; the control bars). GEA 3175 belongs to an incomplete characterised class of NO-containing compounds. For this reason, we analysed the influence of the sGC inhibitor ODQ (2 μM) on GEA 3175. As shown in Fig. 6, the presence of ODQ completely reversed GEA 3175-induced suppression of thrombin-stimulated rises in $[Ca^{2+}]_i$ in platelets. ODQ alone did not affect the response mediated by thrombin.

The influence of GEA 3175 and SNAP on cytosolic Ca^{2+} signalling was further evaluated by analysing thrombin-induced influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular Ca^{2+} stores. The effects of the NO-containing compounds is summarised in Table 1. Fig. 7 shows that thrombin (0.3 u ml^{-1}) caused a rapid rise in $[Ca^{2+}]_i$ in the presence of 0.5 mM EGTA. This response is presumably attributable to liberation of Ca^{2+} from intracellular stores. The subsequent reintroduction of extracellular Ca^{2+} (4 mM) to the suspension of activated platelets also induced a marked cytosolic Ca^{2+} response (Fig. 7B and D). The latter Ca^{2+} signal is assumed to be promoted by depletion of intracellular stores and has been referred to as store-operated Ca^{2+} influx. GEA 3175 (10 μM; incubated for 2 min) significantly reduced thrombin-induced liberation of Ca^{2+} (Fig. 7A and Table 1). This inhibitory effect was abolished when the length of incubation was increased to 30 min (Fig. 7C and Table 1). On the contrary, neither a brief nor a prolonged exposure time of SNAP (10 μM) affected thrombin-stimulated liberation of intracellular Ca^{2+} (Fig. 7A and C). Fig. 7 also shows that both GEA 3175 and SNAP reduced the influx of Ca^{2+} evoked reintroduction of extracellular Ca^{2+}. Interestingly, this inhibitory action persisted after long time of incubation (30 min) with the NO-containing compounds (Fig. 7D and Table 1).

Table 1
The effect of GEA 3175 (10 μM) or SNAP (10 μM) on thrombin (0.3 u ml^{-1})-induced cytosolic $[Ca^{2+}]_i$ increase

		Mn^{2+} quenching	Liberation of Ca^{2+} from intracellular stores	Store-operated influx of Ca^{2+}
		% from control ($n=5$)	$[Ca^{2+}]$ (nM, $n=6$)	$[Ca^{2+}]$ (nM, $n=6$)
Control	2 min		223.9 ± 22.6	518.6 ± 63.6
GEA 3175	2 min	74 ± 1.6[b]	163.1 ± 14.9[a,c]	158.5 ± 22.8[b,d]
SNAP	2 min	79 ± 1.6[b]	231.8 ± 22.9	377.1 ± 41.1[b]
Control	30 min		124.7 ± 11.3	735.7 ± 48.0
GEA 3175	30 min	88 ± 5.0[a]	107.2 ± 7.7	335.4 ± 30.9[b,d]
SNAP	30 min	87 ± 5.1[a]	115.5 ± 9.4	544.2 ± 44.7[b]

Values are presented as means ± S.E.M. Significance of differences between experimental and control groups: [a]$P<0.01$, [b]$P<0.001$, and between the experimental groups: [c]$P<0.01$, [d]$P<0.001$.

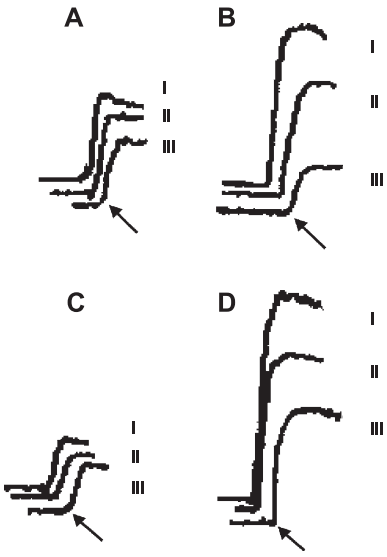

Fig. 7. Effects of GEA 3175 and SNAP on the intracellular mobilization of Ca^{2+} (A and C) and the store-operated influx of Ca^{2+} (B and D). Representative recordings from fura-2-loaded platelets in the presence of 0.5 mM EGTA activated with thrombin (0.3 u ml^{-1}) as indicated by the arrows in A and C. Ca^{2+} ions (4 mM) were added to the cell suspensions 3 min after stimulation with thrombin, as indicated by the arrows in B and D. GEA 3175 (10 μM) or SNAP (10 μM) were added to the platelets 2 min (A and B) or 30 min (C and D) prior to thrombin. Controls, I; SNAP, II and GEA 3175, III. The figure illustrates one of six similar experiments.

The influx of Mn^{2+} across the plasma membrane has been used experimentally as a substitute for agonist-induced Ca^{2+} entry into platelets and other cell types (Sage et al., 1989). We found that 10 μM of GEA 3175 and SNAP significantly reduced thrombin-stimulated induced entry of Mn^{2+} (Table 1). This effect was not influenced on the length of incubation. However, as indicated in Table 1, the magnitude of inhibition was rather small.

3.3. Phosphorylation of VASP

The 46/50 kDa protein VASP is one main substrate protein for cGMP-activated PK-G. As shown in Fig. 8, incubation of platelets for 2 min with 10 μM of GEA 3175 or SNAP resulted in a marked increase in phosphorylation of serine^{239} on VASP. This serine position on the amino acid sequence on VASP is considered to be specific to PK-G. Furthermore, the Western blot shows that the phosphorylation on serine^{239} was equally pronounced after an incubation time of 30 min.

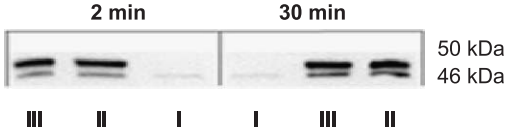

Fig. 8. The effects of GEA 3175 and SNAP on the phosphorylation state, on site serine^{239}, of vasodilator-stimulated phosphoprotein (VASP). Platelets were incubated for 2 or 30 min with GEA 3175 (10 μM; III), SNAP (10 μM; II) or DMSO as control (I). VASP-phosphorylation was analysed by using the antibody 16C2. Immunoblotting was performed by 7.5% SDS-PAGE. Controls, I; SNAP, II and GEA 3175, III. This blot is representative of three independent experiments.

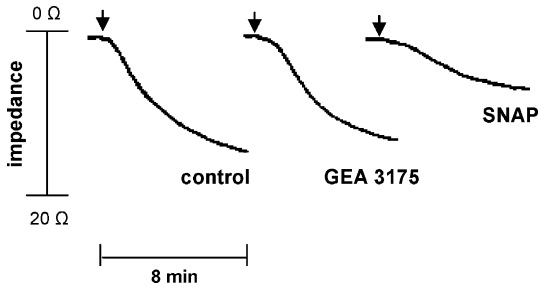

Fig. 9. ADP-induced whole blood aggregation. Aggregation was induced by ADP (40 μM) (arrows). GEA 3175 (30 μM) or SNAP (30 μM) was added 2 min before ADP. The traces are representative of seven similar experiments.

3.4. Effect of SNAP and GEA 3175 on whole blood aggregometry

In heparinized and diluted whole blood, the addition of ADP (40 μM) provoked platelet aggregation (Fig. 9). SNAP (30 μM), but not GEA 3175 (30 μM), significantly decreased the maximal aggregatory response (Fig. 10A). The influence of GEA 3175 (30 μM) and SNAP (30 μM) on thrombin-receptor activated peptide (TRAP; 10 μg ml^{-1})-stimulated aggregation is shown in Fig. 10B. None of the compounds significantly affected TRAP-stimulated aggregation.

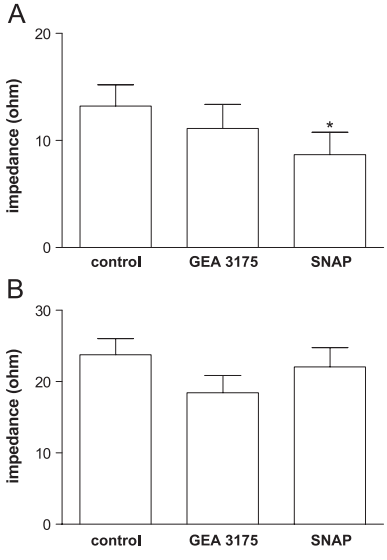

Fig. 10. Summarised effects of GEA 3175 (30 μM) and SNAP (30 μM) on ADP (40 μM; A)-induced or TRAP (10 μg/ml; B)-induced whole blood aggregometry. Statistical significance was tested with ANOVA followed by Newman–Keuls multiple comparison test as post hoc test. See Fig. 2 ($n = 6–7$).

4. Discussion

Mesoionic 3-aryl substituted oxatriazole derivatives represent an incomplete characterised class of NO-containing drugs with smooth muscle relaxant, anti-platelet and anti-proliferative activities (Grenegård et al., 1996; Johansson Rydberg et al., 1997; Lahteenmaki et al., 1998). In this study, we compared the efficacy on human platelets of one of these compounds, GEA 3175, with that of the commonly used *S*-nitrosothiol, SNAP.

We registered similarities, but also significant differences, between these chemically unrelated, NO-containing drugs. First of all, GEA 3175, opposite to SNAP, did not spontaneously release detectable amounts of NO in physiological saline. NO-donors, such as *S*-nitrosothiols, can decompose spontaneously and generate NO^+ (Ignarro et al., 2002), but the liberation of NO may also be accelerated by the presence of Cu(I) ions or certain amino acids (e.g., cysteine) and by enzymatic catalysis (Megson, 2000). GEA 3175 has been reported to be a slow NO-releaser compared to SNAP, and L-cysteine has also been shown to enhance the release of NO from GEA 3175 (Karup et al., 1994). However, GEA 3175 failed also to induce NO-release in the presence of human plasma and isolated human platelets. There are several methods available to detect, directly or indirectly, NO, and some are certainly more sensitive than a NO-microsensor. Therefore, we do not exclude that the oxatriazole derivative releases small amounts of NO. However, considering NO-releasing properties, we conclude that GEA 3175 is a much weaker releaser compared to the *S*-nitrosothiol SNAP.

It has earlier been shown that GEA 3175 is more effective at lower concentrations than SNAP, referring to, for example, the inhibition of human lymphocyte proliferation (Kosonen et al., 1997), inhibition of E-selectin expression and neutrophil adhesion to human endothelial cells (Kosonen et al., 2000) and relaxation of bovine isolated bronchioles (Hernandez et al., 1998). In our study, low micromolar concentrations of GEA 3175 and SNAP significantly elevated cGMP content in human platelets. The dose–response studies indicated that the drugs, after 2-min incubation, were almost equipotent. Consequently, spontaneous or enzymatic release of NO is presumably not a prerequisite for GEA 3175-induced elevation of cGMP. Despite this, time studies revealed a marked discrepancy in cGMP-elevating capacity. The *S*-nitrosothiol was, compared to the oxatriazole derivative, more effective in stimulating cGMP biosynthesis after short time (5 and 20 s) of incubation. This may reflect the extensive liberation of NO from SNAP. After longer time of exposure (2 and 30 min) with SNAP or GEA 3175, the cGMP response was stabilised at lower (although still significantly higher than controls) and equivalent levels. This decrease in cGMP content, seen after prolonged incubation of especially the *S*-nitrosothiol, is likely due to increased activity of phosphodiesterases (Mullershausen et al., 2001).

There is a large body of evidence that NO-containing drugs inhibit agonist-induced $[Ca^{2+}]_i$ rises in platelets (Schwarz et al., 2001). Most likely, this effect is mediated via the elevation of cGMP (Schwarz et al., 2001), and it certainly represents a main molecular mechanism responsible for inhibition of platelet functions. However, cGMP-independent actions of some NO-donors, like GEA 3162 (1,2,3,4-Oxatriazolium,5,-amino-3(3,4-dichlorophenyl)-chloride), SNAP and 3-morpholinosydnomine *N*-ethylcarbamide, have also been reported (Lahteenmaki et al., 1998). Our results confirm that SNAP and GEA 3175 significantly suppress thrombin-evoked rises in $[Ca^{2+}]_i$. In the presence of a sGC-inhibitor, the effect of GEA 3175 was abolished. Therefore, we conclude that the Ca^{2+}-lowering capacity of GEA 3175 was due to the increase in platelet cGMP level. Similar results have previously been published regarding *S*-nitrosothiols (Moro et al., 1996). Our results revealed that the Ca^{2+}-lowering capacity of SNAP, and to lesser extent GEA 3175, rapidly declined by increasing the length of incubation. This phenomenon was most pronounced when analysing thrombin-induced release of Ca^{2+} from intracellular Ca^{2+} stores. Actually, SNAP, but not GEA 3175, failed to suppress the liberation of Ca^{2+} from intracellular stores. In contrast to this, both the NO-containing compounds significantly reduced thrombin-induced Ca^{2+} entry, especially store-operated influx of Ca^{2+}. Based on these results, we suggest that NO-containing compounds are more effective in inhibiting influx of extracellular Ca^{2+} than the release of Ca^{2+} from intracellular Ca^{2+} stores. Furthermore, the results indicate that the oxatriazole derivate GEA 3175, compared to SNAP, induced a more sustained inhibition of thrombin-evoked rises in $[Ca^{2+}]_i$ in human platelets. This is in accordance with the long-acting actions of GEA 3175 registered on airway smooth muscle tissue (Johansson Rydberg et al., 1997).

The results strongly indicate that desensibilisation of platelets to NO involve the cytosolic Ca^{2+} signalling pathway. More specifically, the results suggest that the development of tolerance to NO-containing compounds mainly involved the signalling pathway comprising activation of phospholipase-C, formation inositol-1, 4,5-trisphosphate and the subsequent release of Ca^{2+} from intracellular Ca^{2+} stores. Interestingly, we found that both GEA 3175 and SNAP provoked a marked and long-lasting increase in serine^{239} phosphorylation of VASP. This shows that molecular modifications were present after prolonged time of incubation. It has been shown that phosphorylation of VASP correlates with inhibition of platelet, but the relation to cytosolic Ca^{2+} is unclear. In fact, it has been proposed that VASP phosphorylation represents a Ca^{2+}-independent mechanism responsible for inhibition of platelet functions (Aszüdi et al., 1999). The mechanisms involved in the development of tolerance to nitrovasodilators

have been a subject for research for many years. In platelets, it may involve increased activity of phosphodiesterase 5 (Mullershausen et al., 2001). It is possible that the rapid and large release of NO and/or the very high initial levels of cGMP induced by SNAP, but not by GEA 3175, more easily and rapidly provoked desensibilisation mechanisms. Thus, one early and important consequence of that may be reduced effect of NO on agonist-induced liberation of Ca^{2+} from intracellular Ca^{2+} stores. On the other hand, platelet desensibilisation to NO-containing compounds presumably do not involve the pathway comprising cGMP-dependent activation of PK-G and subsequent $serine^{239}$ phosphorylation of VASP.

The blood contains various NO scavengers and, theoretically, a stable NO-containing compound like GEA 3175 may be a more efficient anti-platelet agent than unstable NO-donors like SNAP. However, impedance measurement in heparinized whole blood showed that both compounds were weak inhibitors of TRAP- and ADP-induced platelet aggregation. There are some possible explanations for the lack of inhibitory effects. First of all, aggregation (considering molecular mechanisms: the conformational change of glycoprotein IIb/IIIa to its high affinity, RGDS-binding state) is probably not the most NO-sensitive platelet functions. Moreover, as stated above, blood contains various proteins and other cell types that may decrease the effect of added NO-containing compounds.

In summary, we registered both similarities and dissimilarities between the *S*-nitrosothiol SNAP and the oxatriazole derivative GEA 3175. The most prominent difference was the lack of spontaneous NO-release from the latter compound. Platelets are, in vitro, insensitive to clinically used organic nitrates like glyceryl trinitrate (Ahlner et al., 1985). This is presumably due to the lack of metabolisation and subsequent release of NO from these compounds (Ignarro et al., 2002; Torfgard and Ahlner, 1994). GEA 3175 is, in common with organic nitrates, lipophilic and does not spontaneously release detectable amounts of NO. Despite this, our investigation shows that the oxatriazole derivative elevated platelet cGMP content and inhibited agonist-induced rises in $[Ca^{2+}]_i$ at low micromolar concentrations. For this reason, it is likely that the oxatriazole derivative is more effective than clinically used nitrovasodilators in suppressing platelet functional responses. Whether or not oxatriazole derivatives may be effective in controlling misdirected excessive platelet activation in vivo remains, however, to be determined.

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