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CHARACTERIZATION OF A NEW COMPOUND, S35b, AS A GUANYLATE CYCLASE ACTIVATOR IN HUMAN PLATELETS

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Abstract—The effects of S35b (4-methyl-3-phenyl sulfonylfuroxan), a new phenyl sulfonylfuroxan compound, were investigated on human platelets activated by different agonists. Platelet aggregation evoked by arachidonic acid (AA), collagen, ADP and thrombin was inhibited by the drug in a dose-dependent manner. S35b inhibited the AA-induced increase of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and production of malondialdehyde. A primary action of the compound on cyclooxygenase is unlikely since: (1) U-46619 (15*s*-hydroxy-11,9-[epoxymethano]-prosta-5*Z*,13*E*-dienoic acid, a stable epoxymethano analog of prostaglandin H_2) could not reverse the inhibitory effect of S35b on AA-induced aggregation and $[Ca^{2+}]_i$ increase; (2) U-46619-induced aggregation and $[Ca^{2+}]_i$ rise were inhibited by S35b; and (3) at high collagen concentrations platelet aggregation (which is unresponsive to aspirin under such conditions) was blocked by S35b as well. Thus the drug action is likely to be exerted at an early step of the platelet activation pathway. The elevation in the platelet cGMP level evoked by S35b in a time- and concentration-dependent manner can account for the inhibitory effect: increased cGMP levels could interfere, for instance, with G protein–phospholipase C coupling and subsequent phosphoinositide hydrolysis.

Platelet activation induced by most physiological agents is followed by a rapid hydrolysis of inositol phospholipids by a specific phosphodiesterase (phospholipase C, PLC§). This generates two second messengers, inositol-1,4,5,-trisphosphate and diacylglycerol. These molecules affect divergent pathways of platelet activation: inositol-1,4,5,-trisphosphate causes an increase in $[Ca^{2+}]_i$ and diacylglycerol causes activation of protein kinase C. Each of these events independently results in platelet activation; together they synergistically stimulate a number of platelet responses including secretion and the release of AA from membrane phospholipid stores (for review, see Refs 1 and 2).

The major inhibitory signalling pathways in platelets use the second messengers cAMP and cGMP. Increasing cAMP inhibits agonist-induced rises in intracellular Ca^{2+} concentration [3–5], stimulates Ca^{2+} sequestration [6–8] and decreases agonist-induced stimulation of PLC [9, 10]. Increasing cGMP is also known to inhibit agonist-induced increase in Ca^{2+} concentration [11, 12] and phosphoinositide turnover in platelets [12]. It has been reported that SNP and other nitrogen oxide-containing compounds, which stimulate soluble guanylate cyclase [13], elevate cGMP levels in platelets, and inhibit platelet aggregation and agonist-induced increase of $[Ca^{2+}]_i$ [11, 14–18]. Moreover, "endothelium-derived relaxing factor" (EDRF), shown to be identical to nitric oxide (NO) [19, 20], significantly attenuates the thrombin-induced rise of $[Ca^{2+}]_i$, increases platelet cGMP levels and reduces thrombin-induced aggregation [21–24].

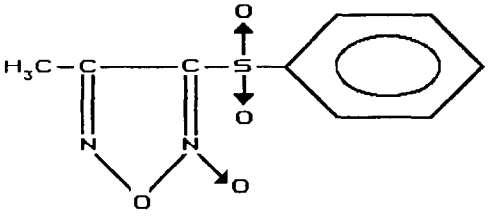

Fig. 1. Chemical structure of S35b (4-methyl-3-phenyl sulfonylfuroxan).

In the present work, we used a newly synthesized compound S35b (4-methyl-3-phenyl sulfonylfuroxan) (Fig. 1) which has been described as a powerful inhibitor of AA-induced platelet aggregation.‖ In order to study the mechanism of action of this drug,

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§ Abbreviations: PLC, phospholipase C; $[Ca^{2+}]_i$, cytoplasmic free calcium; AA, arachidonic acid; SNP, sodium nitroprusside; MDA, malondialdehyde; GSH, reduced glutathione; PRP, platelet rich plasma; DMSO, dimethyl sulfoxide; IBMX, 3-isobutyl-1-methylxanthine; PG, prostaglandin; TCA, trichloroacetic acid.

‖ Calvino R, Fruttero R, Ghigo D, Bosia A, Pescarmona GP and Gasco A, 4-Methyl-3-(*R*-sulphonyl)-furoxans: a new class of potent inhibitors of the platelet aggregation, submitted.

S35b was investigated for its effects: (1) on platelet aggregation induced by different agonists and on AA-elicited MDA production; (2) on reduced GSH levels in resting platelets; (3) on Ca^{2+} movements during agonist-mediated activation; and (4) on the intraplatelet cGMP level.

MATERIALS AND METHODS

Reagents. Human PRP was obtained from AVIS Blood Service (Torino, Italy) within 30 min of collection. AA (Sigma Chemical Company, St Louis, MO, U.S.A.), was dissolved in nitrogen-bubbled absolute ethanol and stored at −20° as 200 mM stock solution. The PG-endoperoxide analog U-46619 (15*s*-hydroxy-11,9-[epoxymethano]-prosta-5*Z*,13*E*-dienoic acid; Cayman Chemical Co., Ann Arbor, MI, U.S.A.) was prepared as 1 mM stock solution in absolute ethanol and stored at −20°. Thrombin (Sigma) was dissolved in isotonic saline and stored at −20° as 100 U/mL stock solution. Quin2 acetoxymethyl ester was obtained from Amersham (U.K.) and stored as a 20 mM DMSO stock solution at −20°. Ionomycin was from Calbiochem (San Diego, CA, U.S.A.). Aspirin (acetylsalicilic acid), collagen, ADP, IBMX, SNP, hemoglobin and bovine serum albumin were obtained from Sigma. The cGMP radioimmunoassay kit was provided by Amersham. Other reagents were Analar grade or of the highest purity available.

Preparation of platelet suspensions and aggregation studies. Human blood from healthy volunteers who had not taken any aspirin-like drug during the past 2 weeks was collected in 1/10 volume of 3.8% trisodium citrate in plastic tubes. PRP (pH 7.6) was prepared by centrifugation at room temperature for 15 min at 200 *g*. Platelet poor plasma was prepared by subsequent centrifugation at 1500 *g* for 10 min. Aggregation studies in PRP were performed according to the light transmission method of Born in a dual channel aggregometer (Elvi 840, Elvi Logos, Milan, Italy). AA (1 mM), collagen (2–4 μg/mL), ADP (10 μM) and the endoperoxide analog U-46619 (1 μM) were tested as inducers. When indicated, PRP was pretreated for 10 min with 0.4 mM aspirin. S35b, dissolved in DMSO/ethanol, was added to PRP 5 min prior to addition of agonist. Control samples received in parallel the same volume addition of DMSO/ethanol. Identical platelet count in corresponding samples was adjusted. All aggregation studies were repeated at least four times with platelets obtained from different donors.

Determination of platelet GSH and MDA. GSH content was measured in PRP as described previously [25, 26]. MDA production was measured as described previously [27] in 1-mL samples of PRP at times indicated after the addition of AA.

Measurement of $[Ca^{2+}]_i$. Washed platelets were prepared from fresh human blood anticoagulated with 0.15 volumes of ACD (85 mM trisodium citrate, 111 mM dextrose, 71 mM citric acid). PRP (pH 6.8) was obtained by centrifugation at 200 *g* for 15 min at room temperature. When indicated, the platelets were loaded with quin2 by incubating the PRP at 37° for 30 min with 15 μM quin2 acetoxymethyl ester [28] and pelleted by centrifugation at 800 *g* at room temperature for 15 min. The supernatant was discarded and the cells were gently resuspended in a physiological saline containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Na-4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 10 mM glucose, pH 7.4 at 37°. The platelet suspension was adjusted to a density of approximately 2×10^8 cells/mL and kept at room temperature in a plastic tube until use. The experiments with quin2-loaded platelets were carried out within 40 min of final resuspension, as platelet responsiveness was constant during that period. $CaCl_2$ (1 mM) was added and the cells were equilibrated at 37° for about 3 min before addition of agonists. When required, washed platelets were preincubated for 10 min with 100 μM aspirin (the same response pattern was observed in platelet suspensions prepared from PRP pretreated for 10 min with 0.4 mM aspirin). S35b was added to platelet suspensions 5 min prior to addition of agonist; control samples received in parallel the same volume addition of DMSO/ethanol. The final solvent concentration never exceeded 0.1%. Measurements of $[Ca^{2+}]_i$ from the fluorescence of intracellular quin2 were performed as described previously [28] in a Perkin–Elmer LS-5 spectrofluorimeter (Perkin–Elmer Corp., Norwalk, CT, U.S.A.). The fluorimetric cuvette holder was thermostatted (37°) and standard monochromator settings were 339 nm excitation (5-nm slit) and 500 nm emission (10-nm slit). Calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was performed as described previously [28]. F_{max} was obtained by adding a large concentration of calcium ionophore (ionomycin) in the presence of 1 mM external Ca^{2+}. To obtain F_{min}, the dye fluorescence was quenched by addition of 2 mM free Mn^{2+}, which gave just the autofluorescence of the cells. Quin2 release into the medium during incubation with the inhibitors (aspirin, S35b) was negligible and autofluorescence of unloaded cells was only slightly affected.

Measurement of platelet cGMP level. For the determination of platelet cGMP content, citrated PRP was incubated (37°) with S35b or SNP at the concentrations and for the time indicated. In some experiments, PRP was preincubated (10 min) in the presence of 0.5 mM IBMX, an inhibitor of cyclic nucleotide phosphodiesterase. After incubation, ice-cold ACD was added to PRP, the platelets were quickly pelleted, the supernatant was aspirated and 0.5 mL ice-cold 6% TCA was added to the pellet. After freeze-thawing, the cellular extracts were centrifuged at 2000 *g* for 10 min at 4°. TCA was removed from the supernatant fraction by extracting the samples six times with 2 mL water-saturated ether and the samples were kept frozen (−20°) until analysis. The amount of intracellular cGMP was measured with the Amersham cGMP radioimmunoassay system and the results were expressed as pmol of cGMP per 10^9 cells.

RESULTS

Effect of S35b on platelet aggregation, MDA production and GSH levels

When PRP was incubated for 5 min with 1–50 μM S35b, the platelet aggregation induced by AA

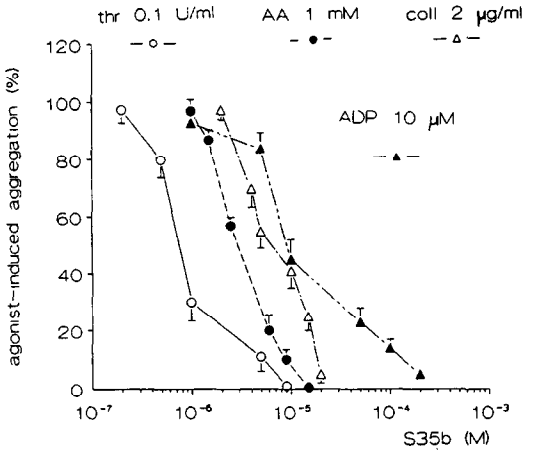

Fig. 2. Concentration–inhibition curves of S35b in AA-, collagen- and ADP-induced aggregation of human PRP, and in thrombin-induced aggregation of human washed platelets. PRP and platelet suspensions (see Materials and Methods) were preincubated for 5 min at 37° in the presence of various concentrations of S35b and then AA (1 mM), collagen (2 μg/mL), ADP (10 μM) or thrombin (0.1 U/mL) was added. The maximal aggregation, reached within 4 min of agonist addition, was expressed as a percentage of the aggregation response induced by agonists in the absence of S35b.

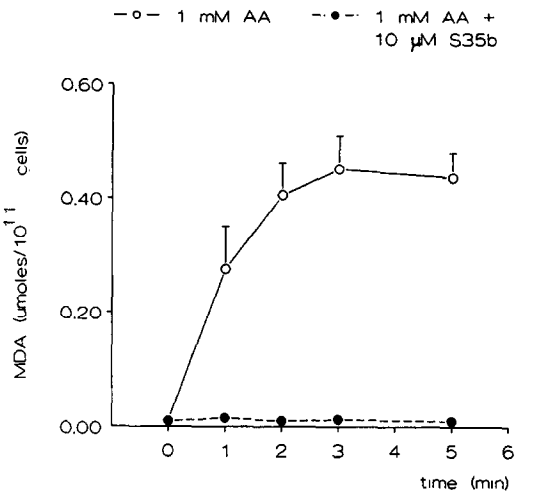

Fig. 3. Inhibition of AA-induced MDA production by S35b. Samples of PRP were preincubated for 5 min with 10 μM S35b. Aggregation was induced by addition of 1 mM AA. After the indicated time the reaction was terminated by addition of TCA and MDA was estimated. Data are mean values ± SD of four experiments.

(1 mM), collagen (2 μg/mL) and ADP (10 μM) was inhibited in a concentration-dependent manner. The maximal aggregation reached within 4 min of addition of the agonist in the presence of S35b was expressed as a percentage of the aggregation response induced by the same agonist in the absence of S35b. The concentration-dependent inhibition curves of S35b are shown in Fig. 2. The apparent IC_{50} values were found to be (μM): 3.6 ± 1.2 (AA), 7.1 ± 3.5 (collagen) and 8.2 ± 2.3 (ADP) (N = 4). When washed platelets were incubated for 5 min with 0.1–10 μM S35b, a dose-dependent inhibition of thrombin (0.1 U/mL)-evoked aggregation was observed and the apparent IC_{50} value was found to be 0.7 ± 0.2 μM (N = 4).

Measurements of MDA production in 1 mM AA-stimulated PRP (0.41 ± 0.08 μmol MDA/5 min/10^{11} cells) showed a complete inhibition of AA-induced MDA formation in PRP pretreated with 10 μM S35b (Fig. 3).

In order to get information on the mechanism of action of S35b, the stable epoxymethano analog of cyclic endoperoxide PGH_2 (U-46619), which stimulates $[Ca^{2+}]_i$ transient rise and aggregation in aspirin-treated platelets [1], was used. S35b-dependent inhibition of AA-induced aggregation was not reversed by repetitive addition of U-46619 (Fig. 4A). Moreover, when 1 μM U-46619 was used to stimulate PRP, S35b inhibited the U-46619-induced aggregation (Fig. 4B). Additionally, when collagen at a high concentration (4 μg/mL) was used to induce aggregation in aspirin-treated PRP, S35b caused a complete blockade of collagen-elicited aggregation (Fig. 4C). These findings suggest strongly that the effect of S35b is independent of the inhibition of PG-endoperoxide synthetase.

The inhibition of AA-induced platelet aggregation by S35b was reversed in the presence of hemoglobin in a dose-dependent manner (Fig. 5). This effect was observed in all experiments, and was present both when hemoglobin was added to PRP together with the inhibitor and when the addition was done some minutes after the aggregatory stimulus. Hemoglobin did not affect the AA response or reverse aspirin-mediated inhibition of AA-induced aggregation (not shown), and could not be substituted by albumin or intact red cells (Fig. 5). A similar effect of hemoglobin was also observed with collagen as the stimulus for aggregation.

In previous work [25, 26] we demonstrated that GSH is an essential cosubstrate for the human platelet PG-peroxidase activity of PG-endoperoxide synthetase. Indeed, GSH depletion blocks the AA-mediated $[Ca^{2+}]_i$ transient rise and aggregation due to inhibition of the production of PGH_2 and/or thromboxane A_2, whose synthesis depends on the availability of GSH [29]. S35b, as a sulfone, could be presumed to exert oxidizing activity [30] on platelet GSH. In order to ascertain whether S35b affected GSH level in platelets, GSH content was measured in S35b-treated PRP. Preincubation of PRP with 1–40 μM S35b did not modify the GSH level in resting platelets: 1.8 ± 0.4 μmol/10^{11} cells.

Effect of S35b on agonist-induced $[Ca^{2+}]_i$ *rise*

Figure 6A represents typical records of calcium movements monitored by quin2 fluorescence changes in platelets stimulated by AA (10 μM) and pretreated for 5 min with increasing S35b concentrations. In the presence of extracellular Ca^{2+}, where the large fluorescence signal is due mainly to Ca^{2+} influx

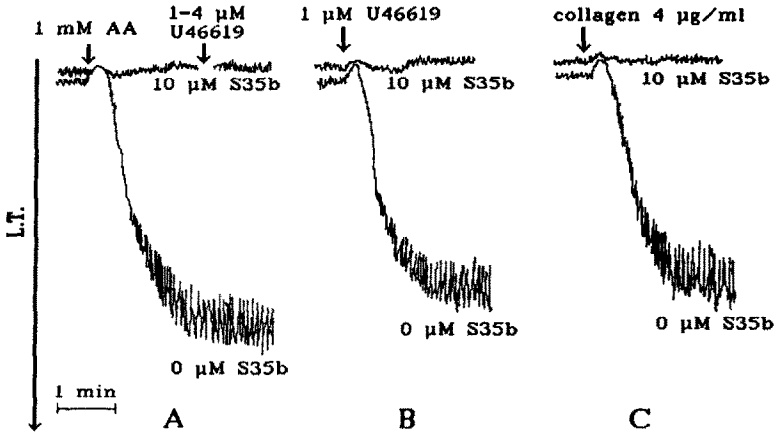

Fig. 4. Human platelet aggregation induced by AA (1 mM, A), U46619 (1 μM, B) and collagen (4 μg/mL, C) in the absence and in the presence of 10 μM S35b. Samples of PRP were treated with S35b for 5 min prior to agonist addition. In C, PRP was pretreated for 10 min with 0.4 mM aspirin. Typical recording representative of six different experiments. L.T., light transmission.

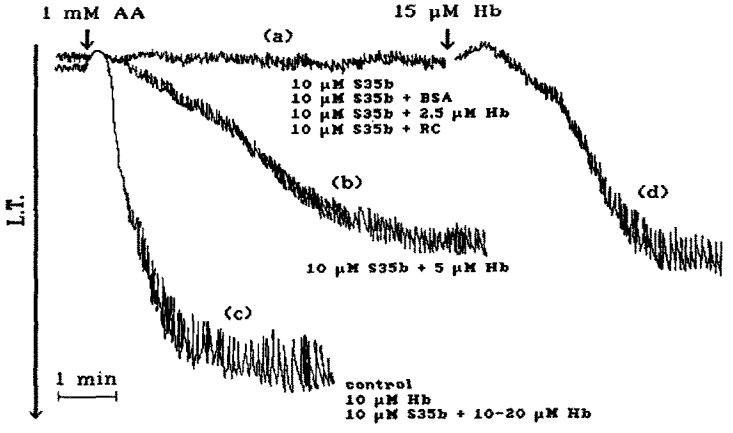

Fig. 5. Dose dependence of the reversing effect of hemoglobin (Hb) on S35b-induced inhibition of AA-elicited aggregation in human PRP. Samples of PRP were treated for 5 min with 10 μM S35b + different doses of Hb or bovine serum albumin (BSA, 15 μM) or intact red cell (RC, with a corresponding amount of Hb) prior to addition of 1 mM AA (traces a, b and c). Trace d was obtained from 10 μM S35b-treated PRP by adding Hb (15 μM) some minutes after the aggregatory stimulus. Typical recording representative of six different experiments. L.T., light transmission.

(reviewed in Ref. 31), S35b promoted a dose-dependent inhibition of AA-induced $[Ca^{2+}]_i$ rise, with no alterations in the basal $[Ca^{2+}]_i$. The same effect was observed when thrombin was used as an agonist: in the presence of external Ca^{2+}, the level of $[Ca^{2+}]_i$ obtained during thrombin (0.1 U/mL) stimulation decreased from 1.012 ± 0.237 μM with thrombin alone to 0.325 ± 0.085 μM with the lowest dose of S35b used (1 μM). Addition of 1 μM U-46619 to platelet suspensions pretreated with 100 μM aspirin (which inhibited completely the AA-mediated $[Ca^{2+}]_i$ rise) elicited a full $[Ca^{2+}]_i$ response, which was totally blocked by S35b (Fig. 6B).

Effect of S35b on platelet cGMP levels

Cellular cGMP levels were measured in platelets following treatment of PRP with S35b. The addition of S35b to unstimulated platelets elicited a dose- and time-dependent increase in cGMP content (Fig. 7 A and B) even in the absence of cyclic nucleotide phosphodiesterase inhibition. The maximal accumulation (100 μM S35b), reached by 10 min, corresponded to a 15-fold increase in cGMP concentration above the baseline. S35b at 10 μM elicited a 5 to 6-fold increase after 10 min incubation. A large amplification of S35b-induced cGMP production was observed in IBMX-treated cells (Fig. 7 A and B). The effect of 1 mM SNP on cGMP level after 5 min incubation in PRP is shown in Fig. 7B. Neither S35b nor SNP had any significant effect on the platelet cAMP level (not shown).

DISCUSSION

This report describes the effect of a newly

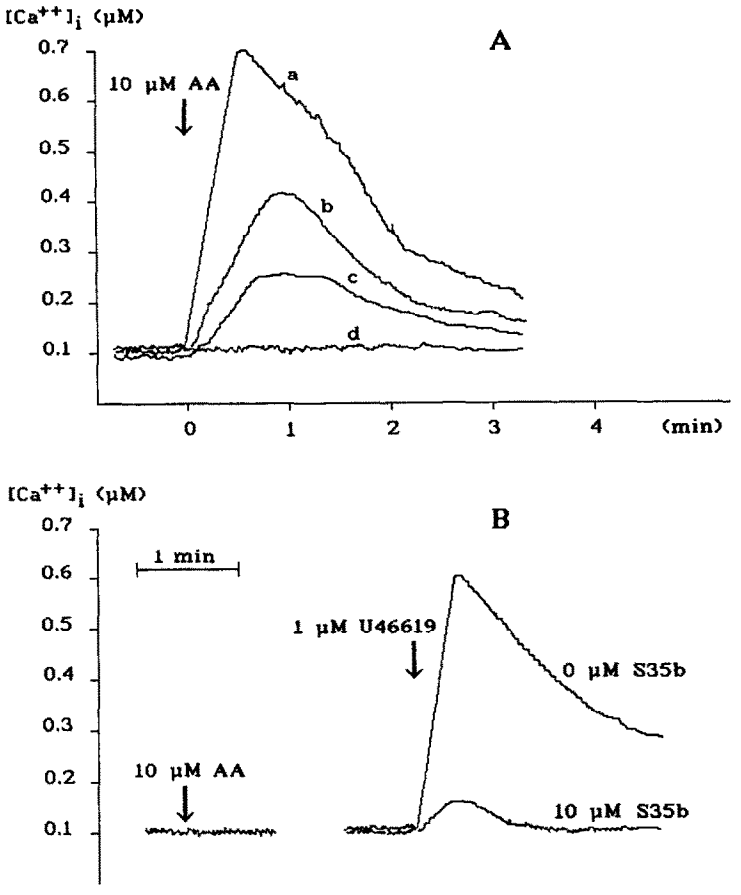

Fig. 6. Inhibition of AA- and U46619-induced increase in $[Ca^{2+}]_i$ by S35b. Suspensions of quin2-loaded (see Materials and Methods) washed platelets (2×10^8 cells/mL) were placed in fluorimeter cuvettes. $CaCl_2$ at 1 mM was added and the cells were equilibrated at 37° prior to agonist addition. When indicated aspirin-treated platelet suspensions were prepared from PRP pretreated for 10 min with 0.4 mM aspirin. (A) Quin2-loaded platelets were incubated with different concentrations (5, 10, 20 μM) of S35b for 5 min and then exposed to 10 μM AA. S35b had no effect on the basal $[Ca^{2+}]_i$, 0.115 ± 0.007 (N = 10). Control platelets maximal $[Ca^{2+}]_i$ response, 0.680 ± 0.12 (N = 10). Each tracing represents a typical quin2 fluorescence recording, in response to a given treatment, out of four experiments with similar results. Calculated values for $[Ca^{2+}]_i$ are indicated on the left. The $[Ca^{2+}]_i$ is plotted on a linear scale. (B) Aspirin-treated platelets, no response to AA. Maximal $[Ca^{2+}]_i$ response to U-46619, 0.595 ± 0.034 μM. Response to U-46619 was additionally measured after 5 min treatment with 10 μM S35b. Each tracing represents a typical quin2 fluorescence recording, in response to a given treatment, out of four experiments with similar results. Calculated values for $[Ca^{2+}]_i$ are indicated on the left. The $[Ca^{2+}]_i$ is plotted on a linear scale.

synthesized compound, S35b, on various platelet responses. S35b is a very effective inhibitor of AA-, collagen-, and ADP-induced aggregation in PRP, and of thrombin-induced aggregation in washed platelets (Figs 2 and 3). Additionally, S35b blocks the aggregatory response elicited by U-46619, a stable epoxymethano analog of cyclic endoperoxide PGH_2 [32]. S35b affects platelet activation by inhibiting the agonist-induced elevation of platelet cytosolic free Ca^{2+} (Fig. 6), which is one of the main intracellular signals mediating platelet responses [31].

S35b-mediated prevention of AA-induced irreversible aggregation and MDA production suggests a possible primary action on cyclooxygenase. This however seems unlikely for the following reasons: (1) S35b-dependent inhibition of AA-induced aggregation and $[Ca^{2+}]_i$ response is not reversed by addition of U-46619, which is able to stimulate $[Ca^{+2}]_i$ transient rise and aggregation in aspirin-treated platelets [1, 29]; (2) S35b inhibits U-46619-induced aggregation and $[Ca^{2+}]_i$ rise; and (3) S35b inhibits collagen-induced aggregation in aspirin-treated platelets at agonist concentrations which are known to induce a platelet aggregation which is not diminished by cyclooxygenase blockers [1]. S35b, as a sulfone [30], could be presumed to exert an oxidizing activity on platelet GSH, which is an essential cosubstrate for the PG-peroxidase activity of PG-endoperoxide synthetase. However, pre-

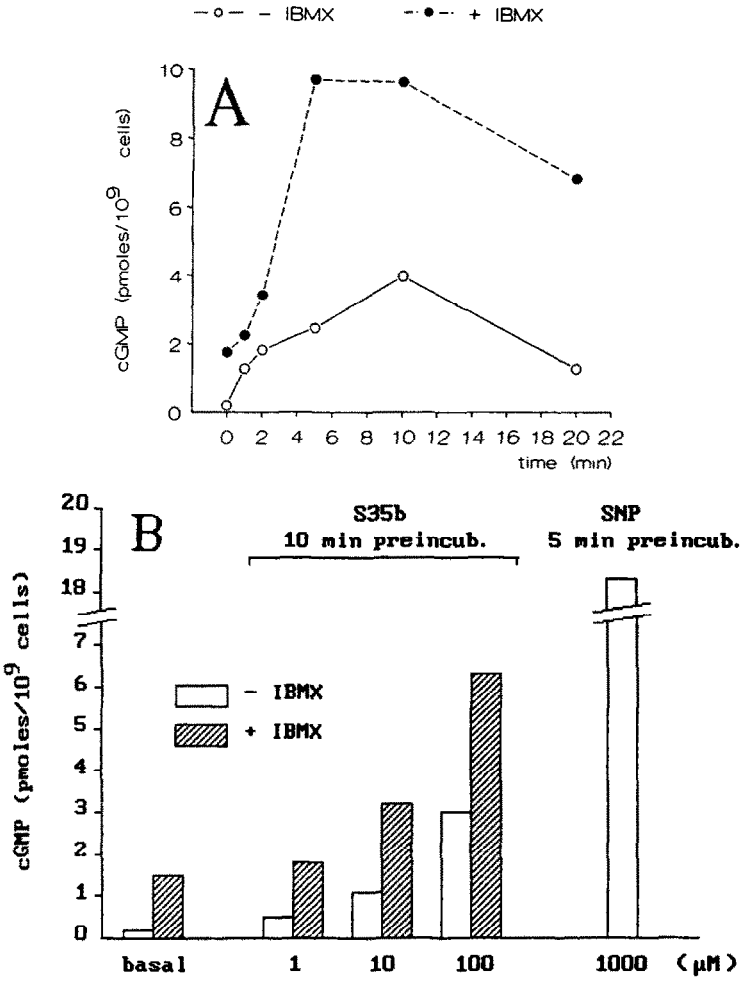

Fig. 7. Time- (A) and concentration- (B) dependence of cGMP accumulation in human platelets treated with S35b in the absence and in the presence of the phosphodiesterase inhibitor IBMX (0.5 mM, 10 min preincubation). Human PRP was treated with 100 μM S35b for different times (A) or for 10 min with different S35b concentrations (1, 10, 100 μM, B). The effect of SNP on cGMP level after 5 min incubation is also shown in B. The intracellular levels of cGMP were measured by radioimmunoassay as described in Materials and Methods. Data are means from two separate experiments performed in triplicate.

incubation of PRP with high S35b concentrations does not modify platelet GSH levels.

The inhibitory effect of S35b may possibly involve an early step in the signal transduction pathway of platelet activation. Indeed, the most interesting finding of this study is that the antiaggregatory effect of S35b is associated with a marked increase in platelet cGMP, which can explain the inhibition of agonist-induced $[Ca^{2+}]_i$ elevation. It has been reported that SNP and other nitrogen oxide-containing compounds, which stimulate soluble guanylate cyclase [13], elevate cGMP levels in platelets and inhibit platelet aggregation [11, 14–18]. Moreover, SNP and 8-bromo-cGMP were shown [11, 12, 17, 33] to inhibit the agonist-induced elevation of platelet $[Ca^{2+}]_i$. So far, elevation of platelet cGMP levels has been proposed as a mechanism by which agents such as NO/endothelium-derived relaxing factor [19, 20] and nitrovasodilators exert their antiplatelet effect. Platelet responses to NO, i.e. strong inhibition of agonist-mediated Ca^{2+} transient rises and aggregation [24], are preceded by rapid and marked accumulation of cGMP [23] and are abolished by hemoglobin and related hemoproteins that bind NO with high affinity [34]. The inhibitory effects of NO are mimicked by the addition of 8-bromo-cGMP, and NO causes heme-dependent activation of soluble guanylate cyclase purified from human platelets [35]. NO and cGMP can account for the vascular smooth muscle relaxant effects and for the antiaggregatory properties of the nitrogen oxide vasodilator drugs (reviewed in Ref. 36).

The precise site of the inhibitory action of cGMP on platelet activation is unclear, although it appears to be at an early point in the activation cascade. A

blockade of thrombin-induced breakdown of inositol phospholipids has been shown [12, 15] which parallels an inhibition of 47-kDa protein phosphorylation and of ATP secretion [16], together with a blockade of thrombin-induced AA release [37]. More recently, it was demonstrated that the ability of cGMP to inhibit phosphoinositide hydrolysis results from an inhibition of a guanine nucleotide regulatory protein activation, and the interaction between G protein and phospholipase C [38].

Indeed, a common feature of all platelet stimuli whose effect is blocked by S35b is their binding to specific receptors on the platelet surface: the signal emitted from the activated receptor is transduced through the plasma membrane by GTP-binding proteins, and the effector system is PLC-induced inositol phospholipid hydrolysis [1, 2].

As far as the mechanism of guanylate cyclase activation by S35b is concerned, we have had no evidence until now that the compound releases NO, as the rapid and efficient scavenging effect of hemoglobin would suggest. SIN-1, the active metabolite of molsidomine, which was thought previously to stimulate guanylate cyclase as an intact molecule rather than by liberating NO, has been shown recently to decompose rapidly into NO even in the absence of cysteine by a radical separation from its ring-opened form SIN-1A [39, 40]. SIN-1 elicits a significant inhibition of thrombin-induced Ca^{2+} influx and PLC activation in human platelets at a concentration which doubles the cGMP intracellular level [15]. This mechanism seems unlikely for the bis-substituted furoxan ring, while a direct interaction of the intact molecule with guanylate cyclase might occur.

In conclusion, we have shown that the newly synthesized compound S35b is a powerful activator of platelet guanylate cyclase and that, via cGMP, it affects platelet stimulation by inhibiting agonist-induced increase of intracellular free Ca^{2+}. Its mechanism of enzyme activation needs further investigation.

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