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IDENTIFICATION OF SMALL PEPTIDE ANALOGUES HAVING AGONIST AND ANTAGONIST ACTIVITY AT THE PLATELET THROMBIN RECEPTOR

EWA M. RUDA,* ALISON PETTY,* MICHAEL C. SCRUTTON,* DAVID P. TUFFIN† and PAUL W. MANLEY†

*Department of Biochemistry, King's College, Campden Hill Road, London W8 7AH, and †Departments of Biology and Chemistry, G. D. Searle & Co., P.O. Box 48, Lane End Road, High Wycombe HP12 4HL, Bucks, U.K.

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Abstract—Two tripeptide analogues (*N*-[3-methyl-1-*S*[[2-*S*-[(methyl-amino)carbonyl]-1-pyrrolidinyl]carbonyl]butyl-D-analine) (SC40476) and *N*-[3-methyl-*S*-(1-pyrrolidinylcarbonyl)butyl]-D-alanine,ethyl ester, hydrochloride (SC42619)), inhibit aggregation of, and secretion from, human platelets induced by thrombin but cause no significant inhibition of esterolysis or fibrin formation catalysed by this enzyme. Inhibition by SC40476 of the aggregatory response induced by thrombin is incomplete. Neither peptide analogue inhibits aggregation induced by ADP, collagen, vasopressin or 11,9-epoxymethanoprostaglandin H_2 (U-46619). Enhancement of the response is observed when non-saturating concentrations of these agonists are employed. SC42619 causes a parallel shift to the right in the concentration–response curve describing aggregation induced by thrombin. The Schild plot of these data has a slope of 1.05 and the pA_2 is 2.9 ± 0.1. Both SC40476 and SC42619 induce a small but significant decrease in the single platelet content of platelet suspensions.

Neither peptide analogue increases platelet cytosolic [Ca^{2+}] measured using quin 2 or Fura 2. Both analogues cause inhibition of the increase in cytosolic [Ca^{2+}] induced by thrombin. Inhibition by SC42619 is competitive with respect to thrombin when the extracellular [Ca^{2+}] is reduced to <0.1 μM but is non-competitive in the presence of 1 mM Ca^{2+}. SC42619 also inhibits the increase in cytosolic [Ca^{2+}] induced by ADP in the presence of 1 mM Ca^{2+} but not the smaller increase caused by this agonist when the medium contains <0.1 μM Ca^{2+}. SC42619 inhibits Mn^{2+} influx induced by thrombin and ADP.

SC40476 and SC42619 inhibit the enhanced incorporation of [^{32}P] into phosphatidic acid observed on stimulation by thrombin of platelets pre-labelled with [^{32}P]-phosphate. Addition of the peptide analogues alone fails to increase significantly the ^{32}P content of phosphatidate, phosphatidylcholine, phosphatidylserine or phosphatidylethanolamine.

SC40476 causes no detectable hydrolysis of glycoprotein V as detected by release of the proteolytic product (glycoprotein V_{FR}).

The results indicate that SC40476 and SC42619 interact selectively with the platelet thrombin receptor. Both peptide analogues act as effective antagonists for this receptor but also possess weak agonist activity which may also result from interaction with the thrombin receptor. The molecular basis for this latter activity has not been defined. SC42619 non-selectively inhibits Ca^{2+} influx induced by several agonists but this effect does not appear to contribute to the observed inhibition of the aggregatory and secretory responses.

Thrombin is a seryl protease which has a central role in the control of the haemostatic system. Although it is best known as the enzyme responsible for the proteolytic cleavage of fibrinogen to yield fibrin monomers [1], thrombin also causes activation of other haemostatic, e.g. Factor V [2], Factor VIIIC [3], Factor XIII [4], and anticoagulant, e.g. Protein C [5], factors as a consequence of peptide bond cleavage. In addition thrombin stimulates many of the cells which are implicated in haemostasis, e.g. platelets [6] and endothelial cells [6]. For the platelet, stimulation by thrombin causes aggregation, secretion and thromboxane A_2 production [6] associated with a number of well-characterised intracellular events. These include phosphoinositide hydrolysis [8], production of 1-2,diacylglycerol, phosphatidate and inositol-1,4,5-trisphosphate [9–11], elevation of cytosolic [Ca^{2+}] as a consequence both of enhanced influx and of intracellular mobilisation of this cation [12] and increased protein phosphorylation [13]. Such events are also observed as a consequence of stimulation with excitatory agonists, e.g. platelet-activating factor [14, 15] and thromboxane A_2 [16, 17], which do not possess protease activity. These and other considerations [18–22] have suggested that proteolysis is not required for activation of platelets by thrombin, which rather may act via a classical receptor occupacy mechanism. Other observations have, however, been interpreted as indicating involvement of proteolysis in stimulation of platelets by thrombin. For example modification of thrombin at the active site seryl residue abolishes both proteolytic activity and also the ability to induce platelet aggregation and secretion [23] although some responses, e.g. enhancement of adhesion to collagen and cytoskeletal modification [24, 25] do not appear to be affected. Furthermore other responses, e.g. amine storage granule secretion

[26], become irreversible after brief exposure of platelets to thrombin, and a surface membrane glycoprotein (glycoprotein V) has been identified which is cleaved by this enzyme [27]. Hence an essential role for proteolysis in platelet activation by thrombin has been proposed [27] although more recent data [18–22] have cast doubt on this postulate.

If platelet activation by thrombin is explicable on the basis of a simple receptor occupancy model, it should be possible to identify small peptides or peptide analogues which have efficacy as agonists for this receptor. Such studies should also define compounds having selective receptor antagonist activity. We describe here the identification of two compounds which have such properties and the characterisation of the ability of these compounds to mimic or inhibit certain responses induced by thrombin. The results obtained, which have been reported in part elsewhere [28] cast further doubt on the essential role of proteolysis of glycoprotein V in cellular signal transduction from the platelet thrombin receptor.

MATERIALS AND METHODS

Preparation of platelet-rich plasma. Blood was obtained by antecubital venepuncture from healthy human volunteers and taken into 0.1 vol. acid–citrate dextrose as the anticoagulant to give a final blood citrate concentration of 10 mM. Platelet-rich plasma (PRP) was prepared by centrifugation at 200 *g* for 20 min and 20°. Residual contamination of PRP by erythrocytes and leucocytes was reduced by further centrifugation at 200 *g* and 20° for 5 min. Acetylsalicylate (100 μM) was then added to the PRP, which was stored at 37°.

Preparation of platelet suspensions. PRP was prepared as described, except that the blood was taken into 0.16 vol. acid–citrate dextrose anticoagulant to give a final blood citrate concentration of 23 mM. Acetylsalicylate (20 μM) and prostaglandin I_2 (PGI_2) (1 μM) were added to the PRP. Platelets were removed from plasma by centrifugation at 350 *g* and 20° for 20 min and the pellet was resuspended in an equal volume of a modified Tyrode's medium, which contained 0.15 M NaCl, 5 mM Na^+–Hepes, 0.42 mM NaH_2PO_4, 6 mM $NaHCO_3$, 2.7 mM KCl, 1.0 mM $MgCl_2$, 5.6 mM glucose and 0.02 U/ml hirudin and had previously been warmed to 37°. The resulting platelet suspension was stored at room temperature.

Synthesis of SC42619. *N*-(1-(*R*)-ethoxycarbonylethyl)-(*S*)-leucine (2.00 g, 0.0086 mol.), triethylamine (2.60 g, 0.026 mol.) and pyrrolidine (0.62 g, 0.017 mol.) were dissolved in dimethylformamide (DMF) (20 ml) and cooled to 0°. After addition of diphenylphosphorylazide (3.55 g, 0.013 mol.) the mixture was allowed to reach room temperature and stirred for 18 hr. Following removal of DMF by rotary evaporation, the residue was washed with chloroform (200 ml) and water (1 l.), before drying over anhydrous sodium carbonate. The crude product was chromatographed on silica (silica gel 60, 70–230 mesh, Merck cat. no. 7734), using chloroform as eluant, to yield a yellow oil, which was dissolved in diethyl ether (20 ml) and heated to 50°. Ethereal hydrogen chloride was added dropwise, until the ether vapour was found to be acidic. The resulting cloudy oil was treated with dichloromethane until a clear solution was obtained. Thereafter the product was allowed to crystallise, yielding SC42619 (0.204 g, 7% yield; melting point 130–133°), which was analysed for purity by elemental analysis and by nuclear magnetic resonance using a Bruker WM 250 spectrometer. The structures of SC42619 and the related compound, SC40476 are shown in Fig. 1.

Measurement of platelet aggregation. Measurement of platelet aggregation as an increase in light transmittance was performed in the presence of 1 mM Ca^{2+} and 1 mg/ml fibrinogen as previously described [29] using a Payton Instruments dual-channel aggregometer interfaced with a Rikadenki DBE-2 dual channel chart recorder. Aliquots of platelets, suspended either in plasma or in the Tyrode's medium, were warmed to 37° in siliconised glass

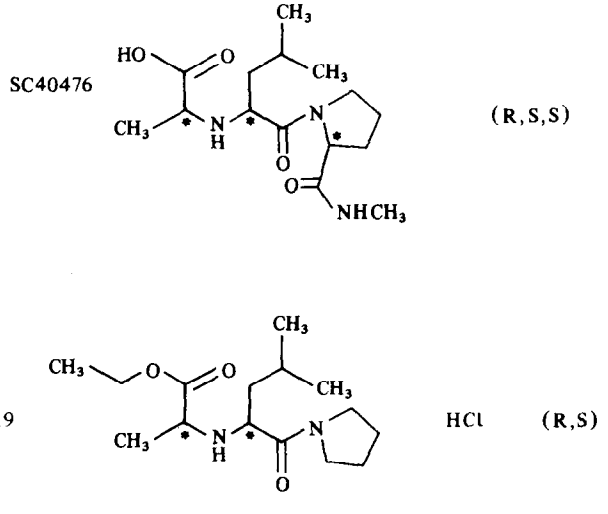

Fig. 1. Structures of SC40476 and SC42619.

cuvettes and pre-incubated in the absence or presence of the peptide for 120 sec before addition of the appropriate agonist.

Aggregation measured as disappearance of single platelets was estimated in the presence of Ca^{2+} and fibrinogen as described above. However, 30 sec after the addition of the agonist the response was terminated by addition of 4 vol. of 1% (v/v) glutaraldehyde. The single platelets remaining were then counted using a Coulter Counter Z_B1 equipped with a 70 μm window [30].

Measurement of ATP secretion. Secretion of ATP was measured as previously described [30]. A modified luciferin-luciferase reagent was added 1 min prior to agonist addition. This preparation has been shown to cause no significant change in responsiveness to thrombin [31].

Measurement of changes in platelet cytosolic $[Ca^{2+}]$. PRP was incubated for 30 min at 37° with Quin-2 acetoxymethyl ester (15 μM) or Fura 2 acetoxymethylester (1 μM) before preparation of the platelet suspension as described above. Changes in light emission at 500 nm were followed in a Perkin-Elmer LS-5 luminescence spectrophotometer [32] in the presence of 1 mM added Ca^{2+} and 1 mg/ml fibrinogen. Platelet suspensions were incubated at 37° in the absence or presence of peptide for 120 sec before addition of thrombin. Quantification of Quin-2 or Fura 2 fluorescence changes in terms of the concentration of Ca^{2+} was performed as previously described [32].

Measurement of changes in the $[^{32}P]$ *content of platelet phospholipids.* These studies were performed by a modification of the procedure described by MacIntyre and Pollock [33]. PRP was prepared as described above and the platelets were collected by centrifugation for 25 min at 350 *g* and 20° in the presence of acetylsalicylate (100 μM) and PGI_2 (1 μM). Platelets were resuspended in 0.1 vol. of the modified Tyrode's medium, containing, in addition, 0.2% bovine serum albumin, and for some studies 0.05 U/ml hirudin and PGI_2 (1 μM). The final cell count was 1–5 $\times$ 10^9 platelets/ml. This platelet suspension was then incubated at 37° for 90 min with carrier-free $[^{32}P]$inorganic phosphate (30 μCi/ml). Platelets were then pelleted by centrifugation for 25 min at 350 *g* and 20° and resuspended in 0.5 vol. of the modified Tyrode's medium.

$[^{32}P]$ labelled platelets were pre-warmed to 37° for 120 sec before adding 1 mM Ca^{2+} and 1 mg/ml fibrinogen. Reactions were initiated by addition of peptide and/or thrombin as indicated and terminated after 30 sec by addition of 3.75 vol. of chloroform/methanol (1:2 v/v). Platelet phospholipids were extracted by the method of Bligh and Dyer [34] and the extract dried under nitrogen. The lipids were then immediately re-dissolved in 25 μl chloroform/ 10^8 platelets and chromatographed on silica gel sheets for one-dimensional separation of phospholipids, using chloroform/propionic acid/propan-1-ol/water (50:75:50:20 v/v) as solvent [35]. After either spraying with rhodamine 6G and visualization under UV light or direct visualization by exposure to I_2 vapour, areas corresponding to individual phospholipids were cut out and counted for radioactivity in a liquid scintillation counter.

Measurement of changes in platelet membrane glycoproteins. Washed platelets were prepared as described above and resuspended in the modified Tyrode's medium containing 0.05 U/ml hirudin and PGI_2 (1 μM). The suspension was equilibrated at 4° in the dark for 10 min and then incubated for a further 10 min with sodium metaperiodate (2 mM). The platelets were collected by centrifugation at 350 *g* and 20° for 25 min and resuspended in the modified Tyrode's medium as above and at a count of 1 $\times$ 10^9 platelets/ml. After incubation with sodium $[^3H]$borohydride (1 mCi/ml) for 90 min the platelets were collected by centrifugation as described above and washed by resuspension in 5 times the labelling volume of fresh buffer followed by centrifugation as described above. $[^3H]$ labelled platelets were finally re-suspended in the modified Tyrode's medium containing 0.02 U/ml hirudin at a platelet count of 5 $\times$ 10^8/ml. Under these conditions $[^3H]$ is primarily incorporated into the galactosyl and galactosaminyl residues of surface membrane gylcoproteins [27].

$[^3H]$-labelled platelets were incubated in the presence of peptide or thrombin for 30 min at 37° before centrifugation for 2 min at 8000 *g* at 20°. The $[^3H]$ content of the supernatant fraction was then estimated by liquid scintillation counting. Aliquots of these supernatant fractions were also prepared for electrophoresis by treatment with 0.1% (w/v) SDS and 2% (v/v) 2-mercaptoethanol for 10 min at 100° before electrophoresis on 10 cm $\times$ 6 mm 5% polyacrylamide gels in 0.5 M phosphate buffer pH 7.4. The gels were stained for carbohydrate content by a modification of the method of Eckhardt *et al.* [36]. The alterations to the published procedure consisted of the use of sodium cyano-borohydride (1.25 mg/ml DMSO) (5 ml/gel) in the dansyl hydrazone reduction step, and the use of water (three 10-min washes) in the final destaining. After scanning at 366 nm using a Gilford model 360 automatic recording spectrophotometer equipped with a Gilford model 2410 linear transport the gels were cut into 5 mm sections and solubilised overnight in "Cocktail T" scintillant (BDH) (4 ml) before estimation of $[^3H]$ content by liquid scintillation spectrometry.

Measurement of enzymatic activity of thrombin. The enzymatic activity of thrombin was assayed using one of the natural substrates (fibrinogen) either by visual estimation of the clotting time [37] or using a turbidometric method which depends on the sequestration of small latex beads into the fibrin polymers and measurement of the consequential changes in the light transmittance of the sample [38]. The esterase activities of thrombin and trypsin were estimated using small model substrates (S-2238) (thrombin) and *p*-toluenesulphonyl-L-arginine methyl ester hydrochloride (TAME) (trypsin) as described previously [39, 40].

Materials. $[^{32}P]$-inorganic phosphate (carrier-free) and $[^3H]$-$NaBH_4$ (10 Ci/mmole) were obtained from Amersham International (U.K.); Quin 2-AM from Lancaster Synthesis; Fura 2-AM from Calbiochem (U.S.A.); S-2238 from KABI Diagnostica (Sweden); hirudin from Pentapharm (Switzerland); collagen from Hormon-Chemie (G.F.R.); and luciferin and luciferase from LKB-Wallac (Sweden).

RESULTS

Properties of inhibition of platelet responses by SC40476 and SC42619

The dipeptide analogue (*N*-[3-methyl-1*S*-[[2*S*-[(methylamino)carbonyl]-1-pyrrolidinyl]carbonyl]-butyl]-D-alanine (SC40476), originally synthesised as a putative collagenase inhibitor but found inactive [41], has activity as both an agonist and an antagonist at the platelet thrombin receptor. This activity has been defined in two types of study. First as shown in Fig. 2, SC40476 caused marked inhibition of aggregation induced by thrombin ($IC_{50} = 825 \pm 70\ \mu M$ (N = 3) at 2 nM thrombin). No detectable inhibition of fibrin formation was observed at the highest concentration of SC40476 tested (3 mM) and only a slight but not significant ($P > 0.1$) decrease in the rate of hydrolysis of S2238 occurred at this level (Fig. 2). Figure 2 also includes data for a related compound (*N*-[3-methyl-1*S*-(1-pyrridonylcarbonyl)butyl]-D-alanine, ethyl ester, hydrochloride (SC42619) which is more potent as an inhibitor of aggregation induced by thrombin ($IC_{50} = 425 \pm 70\ \mu M$ (N = 3) at 2 nM thrombin) (Fig. 2) but also has no significant effect on fibrin formation or S2238 hydrolysis over the concentration range shown in Fig. 2 (data not shown). Figure 2 also shows that complete inhibition of aggregation induced by thrombin is obtained on addition of a saturating concentration of SC42619 but for SC40476 inhibition is incomplete with a maximal $74 \pm 7\%$ (N = 3) reduction in the extent of response. The data shown in Fig. 2 were obtained using a washed platelet system. Similar effects were obtained when comparable studies were performed using platelet-rich plasma except that both compounds were less potent presumably due to binding by plasma proteins (data not shown). SC42619 also inhibited aggregation induced by addition of a non-saturating concentration of trypsin (80 nM) over a concentration range similar to that shown in Fig. 2. Over this concentration range SC42619 caused no significant inhibition of the catalytic activity of trypsin as measured by hydrolysis of TAME (data not shown).

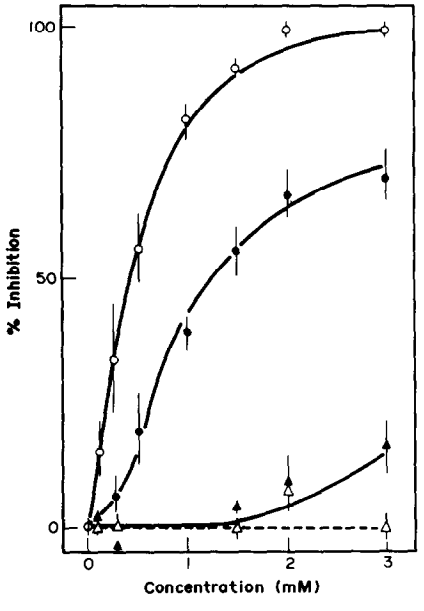

Fig. 2. Effect of SC40476 on amidolytic (▲), clotting (△) and platelet aggregatory (●) activity and of SC42619 on platelet aggregatory (○) activity of thrombin. For measurement of amidolytic and clotting activity thrombin (2 nM) was incubated with SC40476 at the indicated concentration for 2 min at 37° before initiation of the reaction by addition of 50 μM S-2238 (amidolysis) or 0.625 mg/ml fibrinogen (clotting). For measurement of platelet aggregation the washed platelet suspension was incubated for 2 min at 37° with the indicated concentration of SC40476 or SC42619 before addition of thrombin (2 nM). In all cases the data are expressed as % inhibition of the response observed to thrombin in the absence of the peptide. The data shown are the mean values obtained from 3 experiments with determinations performed in triplicate. The bars indicate the SEM.

Second, SC40476 and SC42619, when added at concentrations which gave maximal inhibition of aggregation induced by thrombin, caused no detectable inhibition of this response induced by other excitatory agonists such as collagen, ADP, vasopressin, 11,9-epoxymethanoprostaglandin H_2 (U-46619). When a non-saturating concentration of ADP or U-46619 is employed prior addition of SC40476, or to a lesser extent, of SC42619, enhanced the extent of aggregation observed. This is illustrated in Fig. 3 using U-46619 as the excitatory agonist. The enhancement of the response to a non-saturating concentration of excitatory agonist is particularly marked for U-46619 in the presence of SC40476, where conversion of a reversible to an irreversible response occurred.

The properties of inhibition by SC42619 of aggregation induced by thrombin have been analysed by constructing concentration–response curves for this agonist in the absence and presence of a series of increasing concentrations of the peptide analogue. Addition of SC42619 caused a parallel shift to the right in the thrombin concentration–response curve without any detectable reduction in the maximal extent of the response (Fig. 4A). Analysis of these data as described by Arunlakshana and Schild [42] gave a linear relationship with a slope of 1.05 ± 0.10 ($r = 0.99$; $P < 0.01$ (data not shown)). The pA_2 estimated from this analysis was 2.93 ± 0.10 (N = 3). When ATP secretion, rather than aggregation, was measured, addition of SC42619 also caused a parallel shift to the right in the relationship between the extent of this response and thrombin concentration (data not shown). A meaningful pA_2 could not be determined for SC40476 due to the failure to obtain complete inhibition. However, addition of SC40476 clearly caused a shift to the right in the thrombin concentration response curve (data not shown).

Addition of SC42619 also caused a shift to the right in the concentration–response curve describing the relationship between the rate of aggregation and trypsin concentration (data not shown).

Efficacy of SC40476 and SC42619 as excitatory agonists

Neither SC40476 nor SC42619 induced detectable light transmittance changes of a human platelet suspension as assessed using the optical aggregometer.

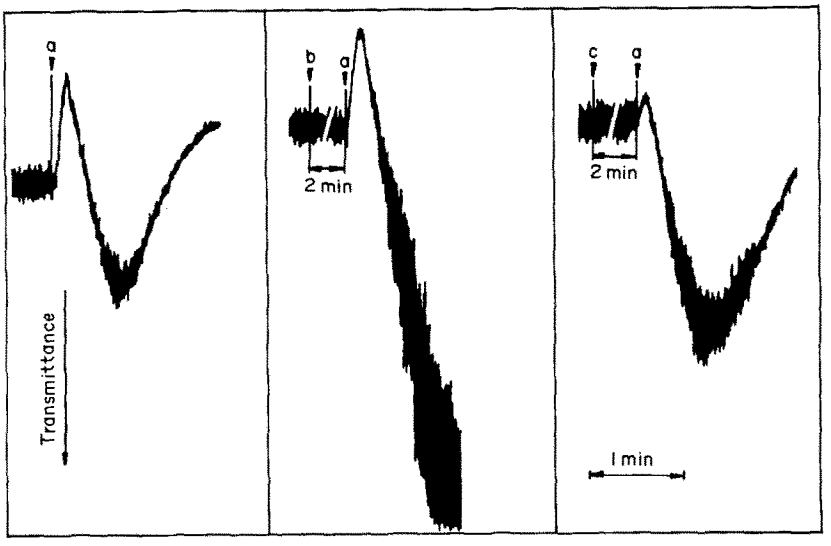

Fig. 3. Effect of SC40476 and SC42619 on aggregation induced by U-46619. Platelet-rich plasma was challenged with a non-saturating dose of U-46619 (2 μM) (added at (a)), following pre-incubation for 2 min in the absence or in the presence of either SC40476 (2 mM) (added at (b)) or SC42619 (2 mM) (added at (c)). The results shown are representative of those obtained in 3 similar experiments.

However, this technique is relatively insensitive and in particular provides no insight into the initial phases of aggregation [43] which can, however, be analysed by measurement of the disappearance of single platelets. Addition of thrombin caused a rapid decrease in the single cell count of a washed platelet suspension which is complete within 30 sec after addition of this agonist and in the presence of a saturating agonist concentration resulted in removal of 80% of the single platelets. The concentration–response curve describing this effect of thrombin (EC_{50} = 0.1 ± 0.04 nM) (Fig. 5) lies to the left of that which describes aggregation measured as an increase in the light transmittance of the suspension (EC_{50} = 0.4 ± 0.1 nM) (Fig. 4A). Addition of SC40476 or SC42619 caused weak but significant aggregation when measured as a decrease in single platelet count. The time course for the response induced by SC40476 or SC42619 was similar to that caused by addition of thrombin but the maximal extent of the decrease in single platelet count was 15–25% with an EC_{50} for both compounds of approximately 0.2 mM (Fig. 5). The data shown in Fig. 5 are corrected for effects of dilution by comparison with the change in platelet count induced by addition of a comparable volume of 0.9% (w/v) saline.

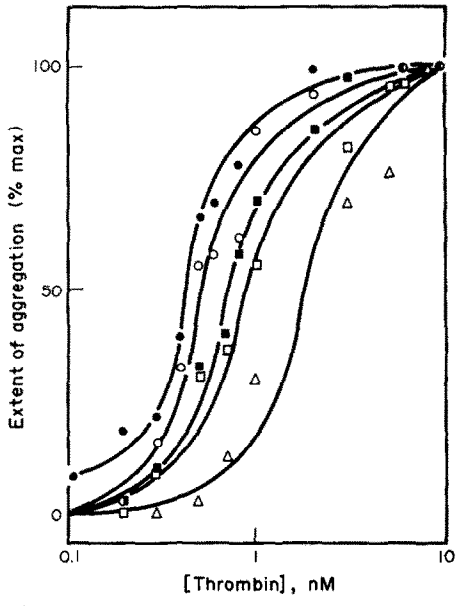

Fig. 4. Effect of SC42619 on the concentration–response curve for aggregation induced by thrombin. Platelets were incubated in the absence (●) or presence of 0.2 mM (○), 0.5 mM (■), 1.0 mM (□) and 2.0 mM (△) SC42619 for 2 min at 37° before addition of thrombin at the concentration indicated. The data shown are representative of results obtained in 3 similar experiments.

Effect of SC40476 and SC42619 on cytosolic Ca^{2+} *concentration and on* $[^{32}P]$ *incorporation into phosphatidate*

We have investigated the basis for the weak agonist activity of SC40476 and SC42619 shown by Figs 3 and 5 by determining whether these compounds could increase cytosolic $[Ca^{2+}]$ or could enhance production of phosphatidate in platelets pre-labelled with $[^{32}P]$-phosphate. The latter assay indirectly indicates the level of 1,2-diacylglycerol present, and hence the activity of phospholipase C [44].

Under conditions where thrombin caused a marked rise in cytosolic $[Ca^{2+}]$ no detectable increase in this parameter resulted from addition of mM concentrations of either SC40476 or SC42619 (Fig. 6B). Similarly neither peptide analogue significantly or consistently enhanced the extent of incorporation of $[^{32}P]$ into phosphatidate or into any of the other major membrane phospholipids, e.g. phosphatidyl-

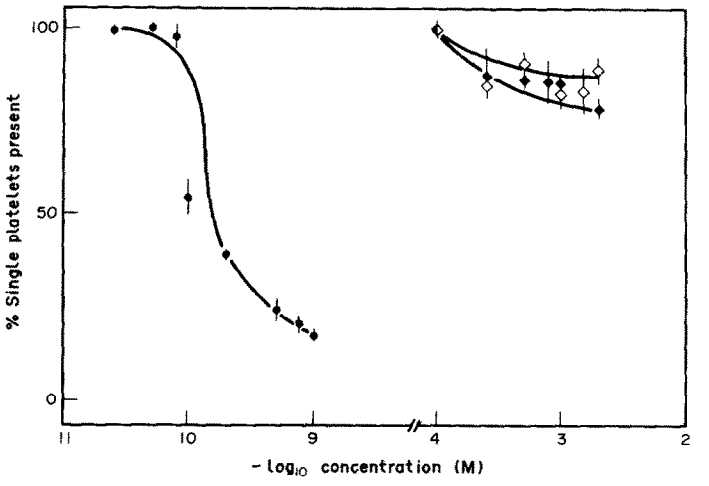

Fig. 5. Aggregation induced by thrombin (●), SC40476 (◆) and SC42619 (◇) as measured by loss of single platelets. The decrease in single platelet count has been corrected for changes induced by addition of an equivalent volume of 0.9% (w/v) saline. The data are expressed as mean ± SEM for three experiments with determinations performed in triplicate.

choline, phosphatidylethanolamine, under conditions in which addition of thrombin markedly increased [^{32}P] incorporation into phosphatidate. However, both peptide analogues consistently inhibited these responses induced by thrombin. This is shown in Fig. 6 for the increase in cytosolic [Ca^{2+}] observed in the presence of both <0.1 μM and 1 mM extracellular Ca^{2+}. Under the former conditions (Fig. 6A) addition of SC42619 caused a shift to the right in the concentration–response curve thus showing a pattern comparable to that observed for inhibition of the aggregation (Fig. 4) and secretion responses. However, in the presence of physiological (1 mM) extracellular Ca^{2+} the predominant effect of SC42619 was to decrease the maximal extent of the increase in cytosolic [Ca^{2+}] induced by thrombin (Fig. 6B). Similarly addition of SC42619 inhibited the increase in cytosolic [Ca^{2+}] induced by ADP when the medium contained 1 mM Ca^{2+} but had no effect when the Ca^{2+} concentration was reduced to <0.1 μM by addition of 1 mM EGTA in the absence of added Ca^{2+}. Such findings suggested that SC42619 acts as a non-selective inhibitor of Ca^{2+} influx in addition to its selective effect as a thrombin receptor antagonist (Fig. 4). This postulate was confirmed using the method of Hallam and Rink [45] which utilises Mn^{2+} as the extracellular cation and detects influx as quenching of intracellular Fura 2 fluorescence. Such quenching can be induced by addition of agonist and results in a decrease in fluorescence to a level below that observed for the unstimulated cell (Fig. 7). This effect is prevented by addition of SC42619 whether ADP or thrombin is used as agonist (Fig. 7).

Inhibition by SC42619 of the increase in [^{32}P] incorporation into phosphatidate induced by thrombin has also been demonstrated (Fig. 8). In this case SC42619 was somewhat more effective (IC_{50} = 0.22 mM at 2.5 nM thrombin) than would have been predicted on the basis of its potency as an inhibitor of aggregation induced by thrombin (Fig. 4).

Effect of SC40476 and SC42619 on the hydrolysis of glycoprotein V in the absence or presence of thrombin

In previous studies glycoprotein V hydrolysis has been quantitated by SDS-gel electrophoresis of the supernatant fraction followed by detection of the cleaved fragment (GPV_{FR}) using either the periodic acid-Schiff reagent and/or autoradiography or fluorography [27]. In order to simplify the procedure we established that simple measurement of [^{3}H] release to the supernatant fraction from [^{3}H]-labelled platelets gave results comparable to those obtained using the more complex procedure. The concentration–response curve for [^{3}H] release induced by thrombin correlated closely with that obtained when release of the fragment (GPV_{FR}) from glycoprotein V was quantitated by SDS-gel electrophoresis followed by staining using the dansylhydrazine reagent (Fig. 9). Co-migration of the single glycoprotein detected on the gel with the major peak of [^{3}H] has also been demonstrated and this analysis further indicated that the molecular weight of this glycoprotein determined from the absorbance trace (87 ± 5 kDa) (data not shown) is in agreement with that reported previously for GPV_{FR} [27]. Figure 9 also demonstrates that SC40476 when added at concentrations adequate to inhibit the aggregatory response induced by thrombin (Fig. 2), and to induce disappearance of single platelets (Fig. 5) caused no detectable hydrolysis of glycoprotein V as measured by [^{3}H] release. Similar results have been obtained when GPV_{FR} was detected on polyacrylamide gels. Further studies showed that addition of SC40476 failed to reduce significantly the extent of glycoprotein V hydrolysis induced by thrombin (data not shown) although the high thrombin concentrations (1–5 nM) required to demonstrate such hydrolysis could make it difficult to detect any inhibition (cf. Fig. 4). Comparable studies using SC42619 have shown that this compound also failed to induce glycoprotein V hydrolysis but when added at 5 mM in the presence of 5 nM thrombin reduced

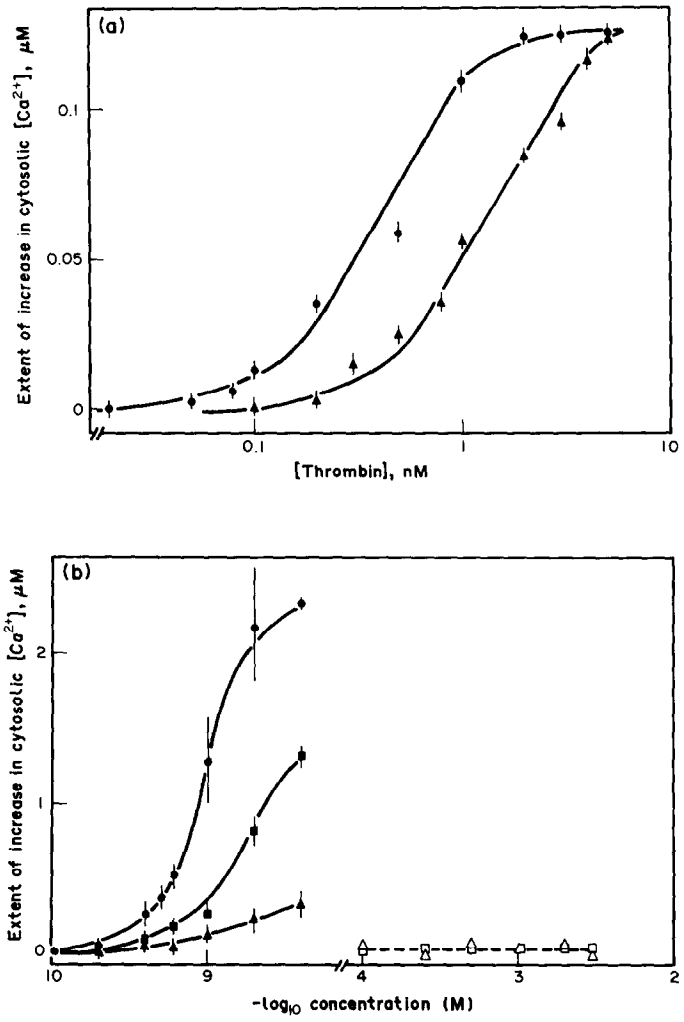

Fig. 6. Effect of thrombin, SC40476 and SC42619 on platelet cytosolic $[Ca^{2+}]$ in the presence of <0.1 μM (a) or 1 mM (b) extra cellular Ca^{2+}. Washed platelets were loaded with either Fura 2 (a) or Quin 2 (b). For addition of SC40476 or SC42619 plus thrombin the platelets were incubated for 2 min at 37° in the absence (●) or presence of 1 mM SC40476 (■) or 1 mM SC42619 (▲) before addition of thrombin at the concentration indicated. The open symbols indicate results obtained on addition of SC40476 (□) or SC42619 (△) in the absence of thrombin. In (a) the extracellular $[Ca^{2+}]$ was reduced to less than 0.1 μM by omission of Ca^{2+} from the suspending medium and addition of 1 mM EGTA immediately prior to the peptide. In (b) 1 mM $CaCl_2$ was added immediately before the peptide. The data are expressed as means ± SEM for triplicate determinations in three experiments. Data similar to those shown in (a) were obtained when platelets were loaded with quin 2 in place of Fura 2.

[^{3}H] release by approximately 50%. Studies of the time course of aggregation and glycoprotein V hydrolysis have shown that single platelet disappearance was complete before any detectable release of GPV_{FR} could be detected (data not shown).

DISCUSSION

The data presented demonstrate that compounds can be developed which impair stimulation of platelets by thrombin without significantly interfering with cleavage by this enzyme of either a low or a high molecular weight substrate (Fig. 2). This profile of activity is unique since other anti-thrombin compounds which have been identified inhibit the catalytic activity of thrombin as well as the effect of this protein as a platelet excitatory agonist [46, 47]. Some studies have suggested that leupeptin might have a profile of activity resembling that described here for SC42619 and SC40476 [48] but we have recently shown that over a similar range of concentration this latter compound inhibits amidolysis, fibrin formation and platelet activation induced by thrombin [49] in a selectivity profile clearly different from that shown in Fig. 2. Such data indicate therefore that leupeptin interacts with the catalytic site on the thrombin molecule itself rather than with the platelet thrombin surface membrane receptor. The region of the thrombin molecule which interacts with the platelet

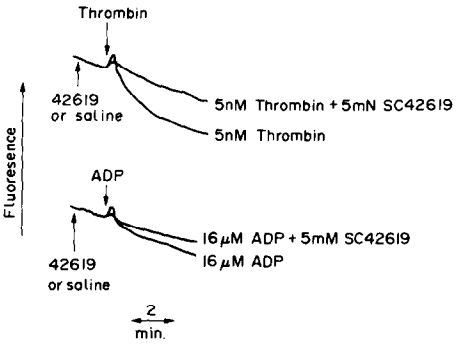

Fig. 7. Effect of SC42619 on Mn^{2+} influx induced by ADP or thrombin. Washed platelets were loaded with Fura and then resuspended in the modified Tyrode's medium containing no added Ca^{2+}. The platelets were preincubated with 1 mM $MnCl_2$ and 5 mM SC42619 (where indicated) for 2 min at 37° before addition of either ADP (16 μM) or thrombin (5 mM). The traces shown are typical of the results obtained in three similar experiments.

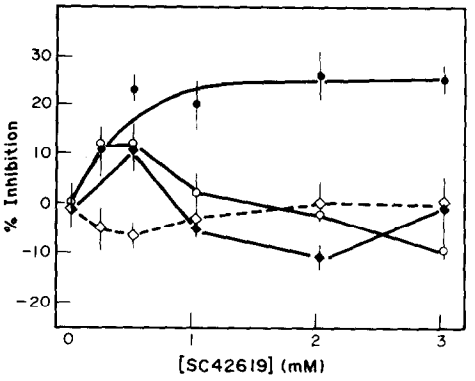

Fig. 8. Effect of SC42619 on changes in the [^{32}P] content of phospholipids in ^{32}P-labelled platelets induced by thrombin. [^{32}P]-labelled washed platelets were incubated with SC42619 at the concentrations indicated for 2 min at 37° before addition of 2.5 nM thrombin. The symbols indicate changes in phosphatidic acid (●), phosphatidylserine (◇), phosphatidylcholine (○) and phosphatidylethanolamine (◆). The data shown are from a single experiment, with determinations performed in triplicate and bars showing SEM. They are representative of the results obtained in 4 other similar experiments.

receptor then appears to be distinct, at least in part, both from the catalytic site of this enzyme and from the macromolecular binding site which interacts with fibrinogen [50]. Furthermore the inhibitory effects of SC42619 and SC40476 on the platelet appear specific for the thrombin receptor since these compounds fail to inhibit aggregation induced by any other excitatory agonist except trypsin. This latter observation accords with the postulate of selective action at the thrombin receptor since the available evidence suggests that aggregation induced by trypsin results from occupancy of this receptor [51]. Our data further demonstrate that SC42619 acts as a competitive antagonist at the platelet thrombin receptor since addition of this compound induces a parallel shift to the right in the concentration–response curve describing aggregation induced by thrombin, and since analysis of these data yields a linear Arunlakshana–Schild plot of unit slope (Fig. 4). The selectivity of action demonstrated for these compounds is somewhat surprising given their relatively low potency ($pA_2 = 2.9$ for SC42619) which has necessitated the use of mM concentrations in most of the studies described. Hence it is not unexpected that our studies have revealed one other effect of SC42619 which, however, is not apparent when analysis is based on studies of functional responses such as aggregation or secretion. The results of Fig. 6 are consistent with the conclusion that SC42619

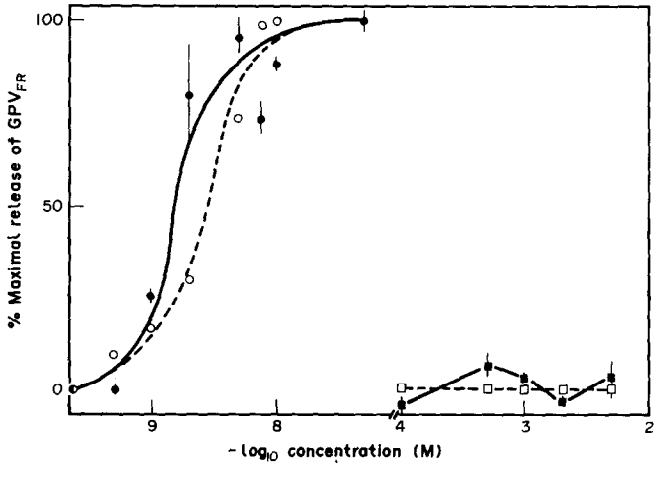

Fig. 9. Effect of thrombin (●, ○) and SC40476 (■, □) on release of glycoprotein V_{FR} from platelets. Cleavage of GPV was estimated both as release of [^{3}H] into the supernatant fraction (●, ■) or as appearance of an 87 kDa glycoprotein band in polyacrylamide gel analysis of the supernatant fraction (○, □). For release of [^{3}H] the data are means ± SEM from 3 experiments with determinations performed in triplicate. For polyacrylamide gel analysis the data are the mean of duplicate determinations in a single experiment and are representative of the results obtained in two other similar experiments.

inhibits Ca^{2+} influx by a mechanism unrelated to the action of this compound at the thrombin receptor. Under conditions where such influx contributes significantly to the observed increase in cytosolic $[Ca^{2+}]$ the extent of inhibition of this latter response by SC42619 is largely independent of thrombin concentration (Fig. 6b). In contrast when Ca^{2+} influx is prevented by decreasing the extracellular $[Ca^{2+}]$ to $<0.1\ \mu M$ inhibition by SC42619 of the increase in cytosolic $[Ca^{2+}]$, which now depends on intracellular Ca^{2+} mobilisation, is overcome by increasing the thrombin concentration (Fig. 6a) and the effect closely resembles that observed for inhibition of aggregation (Fig. 4) and secretion induced by thrombin. In contrast to its effect on aggregation, the inhibition of Ca^{2+} influx by SC42619 is not selective since when this process is monitored indirectly by measurement of Mn^{2+} influx [45] SC42619 inhibits influx induced by both thrombin and ADP (Fig. 7). Furthermore elevation of cytosolic $[Ca^{2+}]$ induced by ADP is inhibited by SC42619 in a medium containing 1 mM Ca^{2+} although no such effect is observed when extracellular Ca^{2+} is reduced to $<0.1\ \mu M$. The properties of inhibition of aggregation by SC42619 therefore correlate with the effect of this compound on the ability of agonists to induce intracellular Ca^{2+} mobilisation and shows no relationship to inhibition of Ca^{2+} influx. This is most clearly seen for thrombin by comparison of Figs. 4 and 6b and for ADP by the fact that no effect on aggregation is associated with clear inhibition of Mn^{2+} influx by SC42619 (Fig. 7). Hence we must conclude that influx of Ca^{2+} is not important for mediation of aggregation or secretion. Such a conclusion seems initially surprising and is at variance with that reached by other workers [52]. However, it is pertinent to note that when intracellular probe buffering is minimised by use of Fura 2 *transient* increases in cytosolic $[Ca^{2+}]$ into the range $0.5–1.0\ \mu M$ can be observed as a consequence of agonist-induced mobilisation of intracellular Ca^{2+} stores [53]. Such a transient rise in cytosolic $[Ca^{2+}]$ might be expected to mediate adequately the rapid aggregation and secretion responses. On the basis of these data we can easily appreciate why other compounds which are known to prevent Ca^{2+} influx would appear ineffective as inhibitors of platelet aggregation or secretion.

Our data also demonstrate that SC40476 and to a lesser extent SC42619 possess weak agonist activity as indicated by the ability of these compounds to decrease the single platelet count (Fig. 5) and to enhance aggregation induced by non-saturating concentrations of agonists other than thrombin or trypsin (Fig. 3). Based on their antagonist properties it seems likely that the agonist activity of SC40476 and SC42619 also results from occupancy of the thrombin receptor. However, this postulate has no direct experimental support. For example no desensitization to thrombin results from pre-incubation with SC40476 (data not shown), although failure to observe this predicted effect is not especially surprising since SC40476 when added alone fails to cause the reversible increase in light transmittance that is required to observe the desensitisation response [54]. Furthermore, neither compound mimics thrombin in increasing cytosolic $[Ca^{2+}]$ (Fig. 6) or reproducibly enhancing $[^{32}P]$-phosphatidate formation. In view of the low level of agonist activity in SC40476 and SC42619 the failure to observe a significant enhancement of $[^{32}P]$ incorporation into phosphatidate is not unexpected since the EC_{50} of this response for thrombin (10 nM) is at least an order of magnitude greater than that characterising aggregation or secretion induced by this agonist ($EC_{50} = 0.4$ nM) (Fig. 4 and data not shown). Similar considerations do not, however, apply to lack of effect on cytosolic $[Ca^{2+}]$ where it should have been possible to detect even a small increase given the high sensitivity of Quin 2 and Fura 2 at the cytosolic $[Ca^{2+}]$ characteristic of the resting cell [12].

If we assume that the agonist activity of SC40476 results from occupancy of the thrombin receptor (see above) then the failure of this compound to cause detectable release of GPV_{FR} (Fig. 9) argues against an *obligatory* role for proteolysis in signal transduction from this receptor. Although this finding is not definitive it adds to a growing body of evidence [18–22] all of which suggests that the enzymatic activity of thrombin is not essential to the ability of this protein to stimulate the platelet. The concentration–response relationship for release of GPV_{FR} by thrombin (Fig. 9) is consistent with this conclusion since comparison of Figs. 5 and 9 shows that near-maximal disappearance of single platelets occurs on addition of a thrombin concentration (1 nM) which causes no detectable release of GPV_{FR}. Similarly maximal release of GPV_{FR} by saturating [thrombin] requires incubation for 30–45 min at 37° whereas single platelet disappearance caused by addition of a much lower thrombin concentration is complete within 30 sec. Such discrepancies may at least in part be rationalised by assuming that release of GPV_{FR} is not an immediate result of peptide bond cleavage (cf. [21]) but it now seems more likely that stimulation of platelets by thrombin can be explained on the basis of a simple receptor occupancy model as is the case for other excitatory agonists.

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