Ligands to the platelet fibrinogen receptor glycoprotein IIb–IIIa do not affect agonist-induced second messengers Ca^{2+} or cyclic AMP

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Previous studies have suggested that the platelet glycoprotein complex GPIIb–IIIa, which is the putative fibrinogen receptor, regulates Ca^{2+} influx into platelets, possibly operating as a Ca^{2+} channel. We have used RGD-peptides (peptides containing the sequence Arg-Gly-Asp; disintegrins), isolated from snake venoms, that have a high affinity and specificity for the fibrinogen-binding site of GPIIb–IIIa to address the question of whether blocking this site inhibits Ca^{2+} movement from the extracellular medium to the cytosol. Using fura-2-loaded human platelets, we found that neither disintegrins nor a monoclonal antibody (M148) to the GPIIb–IIIa complex altered the level of cytosolic Ca^{2+} obtained when the cells were stimulated with various agonists in the presence of either nominal or 1 mM extracellular Ca^{2+} . In the presence of Mn^{2+} , an ion that quenches fura-2 fluorescence, fura-2-loaded platelets were stimulated with thrombin or ADP. Neither disintegrins nor the monoclonal antibody altered the kinetics or the amount of quenching of fura-2 fluorescence by Mn^{2+} . These data indicate that the binding of ligands to the fibrinogen receptor is not associated with an inhibition of Ca^{2+} movement through a receptor-operated channel. Furthermore, the disintegrins have no effect on platelet cyclic AMP metabolism in either the presence or the absence of phosphodiesterase inhibitors.

INTRODUCTION

It is well established that Ca^{2+} is a second messenger mediating, in concert with other second messengers, agonist-stimulated responses in a variety of cells, including human platelets (Rink & Hallam, 1984). Agonist-stimulated increases in platelet cytosolic Ca^{2+} concentrations have been demonstrated using fluorescent indicator dyes such as quin2 and fura-2 (Rink & Hallam, 1984; Simpson *et al.*, 1984; Pollock & Rink, 1986; Daniel *et al.*, 1987). A greater cytosolic Ca^{2+} concentration is achieved in stimulated platelets in the presence of a high concentration of extracellular Ca^{2+} (Hallam *et al.*, 1984a,b; Hallam & Rink, 1985b). Based on this observation, it is postulated that platelets contain receptor-operated Ca^{2+} channels. These channels appear to be distinct from the better-defined voltage-operated channels, since drugs which classically inhibit voltage-operated channels have no effect on the levels of cytosolic Ca^{2+} in stimulated platelets (Doyle & Ruegg, 1985). In addition, Hallam & Rink (1985b) have shown that Mn^{2+} , an ion that quenches the fluorescence of the indicator dyes, can enter platelets through the putative Ca^{2+} channels only when the cells are stimulated with agonists. Recently, Zschauer *et al.* (1988) isolated membrane vesicles from control and thrombin-stimulated platelets. They detected single Ca^{2+} channels in the membranes from stimulated platelets, but, as might be expected if opening of these channels requires agonist-mediated events, they failed to detect Ca^{2+} channels in control membranes.

Glycoprotein IIb–IIIa (GPIIb–IIIa) is a platelet plasma membrane Ca^{2+} -dependent complex which contains both high-affinity binding sites for Ca^{2+} and the receptor for fibrinogen (Fujimura & Phillips, 1983; Phillips & Baughan, 1983; Fitzgerald *et al.*, 1987; Phillips *et al.*, 1988). The fibrinogen receptor activity of the complex is only expressed in activated platelets and is essential for platelet aggregation. Based on studies of platelets from thrombasthenic patients, which lack GPIIb–IIIa, and studies

with complex-specific monoclonal antibodies, it was proposed that the integrity of the GPIIb–IIIa dimer is important for Ca^{2+} homeostasis in unstimulated human platelets (Brass, 1984, 1985; Shattil *et al.*, 1985). Using other monoclonal antibodies directed against GPIIb–IIIa, Powling & Hardisty (1985) investigated the possibility that this complex might serve as a Ca^{2+} channel in stimulated platelets. They found initially that the monoclonal antibody M148 decreased the agonist-stimulated increase in cytosolic Ca^{2+} as measured by quin2 fluorescence in the presence of extracellular Ca^{2+} . However, they found normal changes in cytosolic Ca^{2+} in thrombasthenic platelets, and concluded that GPIIb–IIIa was not a Ca^{2+} channel but that it was possible that another protein closely associated with GPIIb–IIIa might be a channel. Subsequently these authors also demonstrated that the purified Fab-fragment of the same monoclonal antibody had no effect on cytosolic Ca^{2+} (Hardisty & Powling, 1989). Rybak *et al.* (1988) incorporated purified GPIIb–IIIa into phospholipid bilayers and found that the complex enhanced passive diffusion across the liposome membrane. However, more recently, Rybak & Renzulli (1989) demonstrated that Arg-Gly-Asp-Ser (RGDS) had no effect on thrombin-induced Ca^{2+} influx in fura-2-loaded platelets. Sinigaglia *et al.* (1988), in contrast, have presented data suggesting that occupancy of the GPIIb–IIIa complex on platelets by ligands such as fibrinogen and Gly-Arg-Gly-Asp-Ser (GRGDS) reduced ADP- or thrombin-stimulated increases in intracellular Ca^{2+} concentrations.

In the light of a continuing debate concerning the possible role of GPIIb–IIIa in the regulation of agonist-stimulated changes in cytosolic Ca^{2+} concentration, the present experiments were performed to investigate whether disintegrins, a new class of RGD-peptides (peptides containing the sequence Arg-Gly-Asp) from viper venoms (Huang *et al.*, 1987, 1989; Gan *et al.*, 1988; Williams *et al.*, 1989, 1990), have any effect on the component of agonist-induced cytosolic Ca^{2+} increase that is dependent on

Abbreviations used: GPIIb–IIIa, glycoprotein IIb–IIIa complex (fibrinogen receptor); RGD-peptide; peptide containing the sequence Arg-Gly-Asp; PAF, platelet-activating factor; IBMX, isobutylmethylxanthine.

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extracellular Ca^{2+} . We also examined the effect of the disintegrins on cyclic AMP formation, since cyclic AMP is the major second-messenger-mediated inhibitory mechanism in platelets (Mills, 1982), and the elevation of cyclic AMP level is thought to modulate intracellular Ca^{2+} levels. Two disintegrins, elegantin (from *Tremesurus elegans*) and albolabris (from *Tremesurus albolabris*) and the monoclonal antibody M148 (Jones *et al.*, 1984) are all specific high-affinity ligands for the GPIIb-IIIa complex and inhibit fibrinogen binding and platelet aggregation (Huang *et al.*, 1989; Williams *et al.*, 1989, 1990). We found that the inhibitors had no effect on ADP-induced changes in cytosolic Ca^{2+} concentration in the presence of either nominal or 1 mM extracellular Ca^{2+} . In addition, no effect of these inhibitors was seen on thrombin or platelet-activating factor (PAF)-induced changes in Ca^{2+} . We have also shown that disintegrins have no effect on platelet cyclic AMP metabolism; they do not stimulate or inhibit cyclic AMP formation in either the absence or the presence of phosphodiesterase inhibitors.

MATERIALS AND METHODS

Materials

Fura-2 acetoxymethyl ester was purchased from Molecular Probes, Eugene, OR, U.S.A. [^3H]Adenine (31 Ci/mmol) and cyclic [^{14}C]AMP (44 Ci/mmol) were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Iloprost was a gift from Berlex Laboratories, Inc. (Cedar Knolls, NJ, U.S.A.). Forskolin was obtained from Calbiochem (La Jolla, CA, U.S.A.). Prostaglandin E_1 , isobutylmethylxanthine and most other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The monoclonal antibody M148 was generously provided by Dr. Powling and Dr. R. Hardisty, Royal Free Hospital, London. All other chemicals were analytical grade. Echistatin was generously provided by R. Gould and P. Friedman, Merck Sharp and Dohme Research Laboratories, West Point, PA, U.S.A. Arg-Gly-Asp-Ser and the fibrinogen γ -chain dodecapeptide were purchased from Peninsula, Belmont, CA, U.S.A.

Preparation of platelets

Human blood was taken by venipuncture from informed healthy volunteers into acid/citrate/dextrose (Aster & Jandel, 1964). Platelet-rich plasma obtained by centrifugation at 180 *g* for 15 min at ambient temperature was again centrifuged at 800 *g* for 15 min. The platelet pellet was resuspended in 0.2 vol. of autologous platelet-poor plasma and incubated with 5 μM -fura-2 acetoxymethyl ester for 30 min at 37 °C. The platelets were separated from plasma protein and extracellular fura-2 by gel filtration on Sepharose 2B (Pharmacia) using a Ca^{2+} -free Tyrode's buffer containing 0.2 % albumin, 5 mM-glucose, and 5 mM-Hepes (pH 7.3). The cell suspension was adjusted to a final concentration of $(2-3) \times 10^8$ cells/ml.

Purification of albolabrin and elegantin

Both albolabrin (7574 Da) and elegantin (7806 Da) were purified by the method of Williams *et al.* (1989). These are mid-size disintegrins from the viperinae branch of the vipers (Huang *et al.*, 1987; Gan *et al.*, 1988). In brief, 20–40 mg of lyophilized viper venom was dissolved in 0.2–0.4 ml of 0.1 % trifluoroacetic acid at 4 °C, centrifuged to remove insoluble material and submitted to reverse-phase h.p.l.c. on a wide-pore C_{18} silica matrix column (Vydac). The gradient used was 0–80 % acetonitrile containing 0.1 % trifluoroacetic acid. Fractions were tested for inhibition of ADP-induced platelet aggregation in platelet-rich plasma.

Purification of monoclonal antibody M148

Monoclonal antibody M148 (Jones *et al.*, 1984) was isolated as an IgG fraction from ascitic fluid using caprylic acid by a modification of the technique of Steinbuch & Audran (Steinbuch & Audran, 1969; Newman *et al.*, 1985). Briefly, ascites fluid (1 ml) containing monoclonal antibody M148 was diluted with 2 vol. of 0.06 μM -acetic acid, pH 4.0 (adjusted with NaOH), and 100 % caprylic acid was added dropwise to a final concentration of 1.1 % (v/v). The mixture was stirred at room temperature for 30 min and the precipitate was removed by centrifugation. The supernatant was then dialysed extensively at 4 °C against 50 mM- NaPO_4 /150 mM- NaCl , pH 7.4. The dialysed supernatant was tested for its ability to inhibit ADP-induced (10 μM) platelet aggregation of washed platelets in the presence of fibrinogen.

Protein estimations

All protein estimations were performed using a microscale version (Williams *et al.*, 1978) of the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as standard.

Preparation of washed platelet suspensions

Blood collected in acid/citrate/dextrose, pH 6.5, was centrifuged at 150 *g* at room temperature for 10 min to collect platelet-rich plasma. A washed platelet suspension was prepared by the method of Morinelli *et al.* (1987), except that all steps were carried out at room temperature, and suspended in Tyrode's albumin solution, pH 7.35, containing 3.5 mg of bovine serum albumin/ml (Sigma, fraction V).

Estimation of platelet cytosolic Ca^{2+} concentrations

Ca^{2+} measurements were made using the fluorescent indicator dye fura-2. An aliquot (1 ml) of the fura-2-loaded platelet suspension was added to a cuvette which was thermostatically regulated to 37 °C and stirred continuously. Fluorescence measurements made in a Perkin-Elmer LS-5 fluorimeter using an excitation wavelength of 340 nm, or in some cases 360 nm, and an emission wavelength of 510 nm. In order to convert fluorescence measurements to Ca^{2+} concentrations, $F_{\min}$ was determined following the addition of digitonin (50 mM) in the presence of EGTA and Tris base; $F_{\max}$ was measured by the addition of excess CaCl_2 . Free Ca^{2+} was calculated using these values and a K_d of 224 nM (Gryniewicz *et al.*, 1985) after correction for extracellular dye.

Measurement of cyclic AMP formation

Cyclic AMP formation was measured by incubating platelet-rich plasma for 1 h with 1 μCi of [^3H]adenine/ml, followed by washing by centrifugation at 1000 *g* for 15 min and resuspension in Tris-buffered saline (40 mM-Tris/HCl, pH 7.4 with 0.15 M- NaCl chloride) (Ashby, 1988). Assays were performed at 30 °C by addition of 0.5 ml of a suspension of radiolabelled washed platelets to a solution of disintegrins containing forskolin, prostaglandins and adrenaline where appropriate, contained in 50 ml of buffer. Following a 10 min incubation, reactions were stopped by addition of 0.5 ml of stopping solution containing 0.6 M- HClO_4 and 2 % SDS with cyclic [^{14}C]AMP as a recovery standard. The cyclic AMP content of each sample was determined by the method of Salomon (Salomon, 1979; Ashby & Grant, 1987). Results are expressed as percentage of total radioactivity appearing as cyclic AMP. Where indicated, isobutylmethylxanthine (IBMX) was added to the platelet suspension as a 200 mM solution dissolved in dimethyl sulphoxide.

RESULTS

Inhibition of platelet aggregation by disintegrins and monoclonal antibody M148

Fig. 1 shows the effect of albolabrin, a peptide obtained from the venom of the viper *Tremeresurus albolabris*, on the platelet

Fig. 1. Inhibition of platelet aggregation by albolabrin

Washed human platelet suspensions (5×10^8 cells/ml) were stimulated with $10 \mu\text{M}$ -ADP in a Payton aggregometer. Platelet suspensions were treated with iso-osmotic saline (a), or a final concentration of 250 nM-albolabrin (b) or 400 nM-albolabrin (c).

aggregation induced by ADP in the presence of fibrinogen. Albolabrin is an effective and potent inhibitor of platelet aggregation, with an IC_{50} (concn. causing 50% inhibition) for aggregation of 210 nM. Similar results were obtained with both the monoclonal antibody M148 and elegantin, a similar venom from *Tremeresurus elegans*. Inhibition of platelet aggregation was dose-dependent in all cases. The purified IgG fraction (0.74 $\mu\text{g}/\text{ml}$) containing monoclonal M148 and 250 nM-elegantin was required to inhibit the aggregation of gel-filtered platelets.

Effects of disintegrins and monoclonal antibody M148 on agonist-induced platelet Ca^{2+} fluxes

Based on reports in the literature that binding of ligands to the GPIIb-IIIa complex has an influence on Ca^{2+} flux (Powling & Hardisty, 1985; Rybak *et al.*, 1988; Sinigaglia *et al.*, 1988), we studied the effect of the addition of either disintegrins or M148 on the changes in the intracellular Ca^{2+} concentration that occur when platelets are activated with agonists. Changes in intracellular Ca^{2+} were determined in gel-filtered platelets in the presence of 1 mM extracellular Ca^{2+} or nominal Ca^{2+} (approx. 10^{-5} M). The reason for using nominal Ca^{2+} is that under these conditions the Ca^{2+} gradient across the plasma membrane is lowered, and it is possible that a partial blockade of a putative Ca^{2+} channel might be more easily detected. Fura-2 fluorescence traces from one experiment (out of at least five) using ADP as agonist is shown in Fig. 2. A 2-fold excess of peptide or a 10-fold excess of M148 over that required to completely inhibit platelet aggregation had no detectable quantitative or kinetic effects on ADP-induced Ca^{2+} mobilization. Similar results were obtained when either thrombin or PAF were used as agonist (results not shown). In other experiments, different concentrations of

Fig. 2. Effect of monoclonal antibody M148 and elegantin on ADP-stimulated changes in intracellular Ca^{2+} as measured by fura-2

Measurements were made in a Perkin-Elmer LS-5 spectrofluorimeter modified to allow stirring, using an excitation wavelength of 340 nm. In each of the four panels the trace on the left represents the control, to which an appropriate volume of buffer was added. The samples in (a) and (c) were first treated with 1 mM- Ca^{2+} (added at the downward arrow). The samples shown in (a) and (b) (right side) were treated with $50 \mu\text{l}$ of M148 (solid upward arrow). The samples of (c) and (d) (right side) were treated with $10 \mu\text{l}$ of elegantin (final concn. 500 nM; solid upward arrow). All samples were treated with $10 \mu\text{l}$ of 1 mM-ADP (broken arrow).

Table 1. Effects of disintegrins and monoclonal antibody M148 on agonist-induced increases in cytosolic Ca^{2+}

The data are presented as the increase in Ca^{2+} concentration (nM) above the basal level and represent the averages $\pm$ S.D. of four determinations. The data are derived from two separate experiments done on different days. In the case of the data with 1 mM- Ca^{2+} , the basal Ca^{2+} concentration was 40 nM. In the case of the other two sets of data (nominal Ca^{2+} and thrombin), the basal Ca^{2+} concentration was 78 nM. N.D. represents not determined.

	Increase in Ca^{2+} concentration above basal (nM)		
	ADP		
	1 mM- CaCl_2	Nominal Ca^{2+}	Thrombin
Control	131.1 $\pm$ 14.9	109.0 $\pm$ 12.1	457.8 $\pm$ 130.7
M148	132.1 $\pm$ 13.5	120.0 $\pm$ 22.0	N.D.
Elegantin	137.9 $\pm$ 17.1	122.4 $\pm$ 12.8	450.6 $\pm$ 48.6
Albolabrin	135.3 $\pm$ 5.1	110.4 $\pm$ 10.2	N.D.

disintegrin or M148 were again used, with no discernible effect on Ca^{2+} mobilization as measured by fura-2 fluorescence. A set of experiments using ADP and thrombin as agonists were subjected to statistical analysis using analysis of variance and Dunnett's *t* test as a criterion of difference. Table 1 shows the results of this experiment, in which all determinations were made in quadruplicate. No statistical differences were detected between

Fig. 3. Effect of albolabrin on thrombin-induced influx of Mn^{2+}

(a) and (b) are controls to which 10 μl of Tyrode's buffer (solid upward arrow) was added before addition of thrombin. The samples in (c) and (d) were treated with 10 μl of albolabrin (1 μM final concn.; solid upward arrow) 1 min before the addition of thrombin. In (a) and (c), an excitation wavelength of 340 nm was used; in (b) and (d), 360 nm was used. All samples were treated with 10 μl of thrombin (100 units/ml) added at the broken upward arrow and 10 μl of 100 mM- Mn^{2+} added at the solid downward arrow.

Fig. 4. Effects of albolabrin and monoclonal antibody M148 on ADP-induced influx of Mn^{2+}

All measurements were made using an excitation wavelength of 360 nm. Samples from (a) and (b) were treated with 10 μl of Tyrode's buffer (1 min) before addition of Mn^{2+} (results not shown). The sample of (c) was treated with 10 μl M148 (0.74 mg/ml) and that of (d) with 10 μl of albolabrin (1 μM final) 1 min prior to addition of Mn^{2+} . All samples were treated with 10 μl of 100 mM- MnCl_2 . In all cases, addition of Mn^{2+} produced an initial drift, followed by a stabilization. All traces begin after stabilization has occurred. Either buffer (10 μl ; a) or ADP (10 μl of 1 mM; b-d) was added at the solid arrow followed by 10 μl of thrombin (100 units/ml). The horizontal broken lines in (b), (c) and (d) indicate the result obtained in (a) for ease of comparison.

control and treated groups under any of the conditions used in these experiments.

Although these experiments indicated that it was unlikely that occupancy of the fibrinogen receptor had an effect on influx of Ca^{2+} into the platelet cytosol, they were rather indirect and were complicated by the fact that a major portion of the agonist-stimulated Ca^{2+} increase results from mobilization of intracellular stores. A more direct indication of opening of Ca^{2+} channels is based on the observation that Mn^{2+} is able to enter the agonist-stimulated platelet. Entry of Mn^{2+} is easily monitored, since the fluorescence of fura-2 is strongly quenched by Mn^{2+} (Grynkiewicz *et al.*, 1985). Fig. 3 shows representative fluorescence traces using thrombin as an agonist. With this agonist, channels remain open for several minutes and Mn^{2+} can be added after addition of thrombin. In Figs 3(a) and 3(c) the normal excitation wavelength of 340 nm was used, whereas in Figs. 3(b) and (d), 360 nm was used as excitation wavelength. The latter wavelength is the isosbestic point, i.e. it is insensitive to changes in Ca^{2+} and only quenching by Mn^{2+} is seen (Sage *et al.* 1989). The presence of the disintegrin albolabrin did not reduce the Mn^{2+} -induced quenching of the fluorescence when either wavelength was used to excite fura-2. Identical results were obtained with both M148 and elegantin. The results were essentially the same as when Mn^{2+} was added before addition of thrombin. Fig. 4 shows the results of similar experiments (excitation wavelength 360 nm) conducted using ADP (10 μM) as agonist. MnCl_2 was added before addition of ADP, since the opening of divalent channels appears to be more transient when ADP is the agonist (Shattil *et al.*, 1985; Rink, 1988). In all cases, ADP-induced Mn^{2+} influx was not detectably altered by the presence of the monoclonal

Fig. 5. Effects of albolabrin and elegantin on cyclic AMP metabolism in intact platelets

Platelets were prelabelled with [^3H]adenine as indicated in the Materials and methods section and challenged with (a) albolabrin (400 nM final) or (b) elegantin (400 nM final) in the absence or presence of a phosphodiesterase inhibitor (5 mM-IBMX). Experiments were also carried out in the presence of forskolin (100 µM) or iloprost (0.1 µM) to determine whether the disintegrins inhibited cyclic AMP formation induced by these agents. Inhibition of forskolin- or iloprost-stimulated cyclic AMP formation by adrenaline (100 µM) was included as a control. Treatments: 1, no addition; 2, albolabrin (2 µg/ml); 3, elegantin (2 µg/ml); 4, forskolin; 5, forskolin + albolabrin; 6, forskolin + elegantin; 7, forskolin + adrenaline.

antibody or the disintegrin. The subsequent addition of thrombin led to further quenching of the fura-2 by Mn^{2+} influx, demonstrating that although ADP does not cause complete quenching of the dye, opening of additional channels with thrombin allows more Mn^{2+} to enter and completely quenches the fluorescence of fura-2.

Effect of disintegrins on cyclic AMP metabolism in platelets

We examined the effect of the disintegrins on cyclic AMP formation in platelets, since stimulation of cyclic AMP formation is the major second-messenger-mediated inhibitory mechanism in platelets (Mills, 1982). Elevation of the cyclic AMP concentration is thought to modulate intracellular Ca^{2+} levels, and would provide an alternative mechanism for any disintegrin-mediated effects on Ca^{2+} flux. Fig. 5 indicates that neither disintegrin (albolabrin or elegantin) stimulated basal levels of cyclic AMP formation; nor did they augment or inhibit forskolin- or iloprost-stimulated cyclic AMP levels in either the presence or the absence of the phosphodiesterase inhibitor IBMX. In contrast, adrenaline, included as a control, produced significant inhibition of forskolin- and iloprost-stimulated cyclic AMP formation.

DISCUSSION

The classical role of GPIIb-IIIa has been described as a receptor for fibrinogen, mediating platelet aggregation (Phillips *et al.*, 1988). It has also been shown that GPIIb-IIIa in aggregated platelets becomes associated with the platelet cytoskeleton (Phillips *et al.*, 1980), and there is a growing interest in the possible role of integrins in transmembrane signalling, which may be initiated or modified by soluble or matrix-bound adhesive proteins (Ruoslahti & Pierschbacher, 1987; Schindler *et al.*, 1989). For example, fibrinogen binding to platelets appears to promote Na^+/H^+ exchange in adrenaline-stimulated platelets that is inhibited by anti-GPIIb-IIIa monoclonal antibodies which block fibrinogen binding (Banga *et al.*, 1986). Although it is well established that Ca^{2+} is a second messenger in platelets, mediating agonist-stimulated responses in conjunction with other second messengers (Rink & Hallam, 1984), its suggested role in mediating the exposure of the fibrinogen receptor is less well understood (Fujimura & Phillips, 1983; Phillips & Baughan, 1983; Shattil & Brass, 1987; Phillips *et al.*, 1988).

The present studies were stimulated by reports in the literature suggesting a role for GPIIb-IIIa in Ca^{2+} homeostasis in platelets (Brass, 1984, 1985; Shattil *et al.*, 1985), which could be modified by soluble ligands to the glycoprotein complex (Powling & Hardisty, 1985; Rybak *et al.*, 1988). We have no essential disagreement with the work of Brass and coworkers; whereas they were measuring Ca^{2+} exchange in resting platelets, we have examined changes in cytosolic Ca^{2+} in platelets activated by agonists. The effects observed by these authors may be due to changes in bound Ca^{2+} on the cytoplasmic membrane, since it is clear that GPIIb-IIIa does bind Ca^{2+} tightly (Shattil *et al.*, 1985). Furthermore, Brass (1985) demonstrated that antibody binding *per se* had no effect on Ca^{2+} flux, but established a correlation between the preservation of the GPIIb-IIIa receptor (preincubation for 1 h at 37 °C) and the preservation of platelet Ca^{2+} flux. It may be that stabilizing the GPIIb-IIIa receptor, a major membrane component, also indirectly stabilized a Ca^{2+} channel.

Sinaglia *et al.* (1988) reported reduced Ca^{2+} influx into platelets using the peptide Gly-Arg-Gly-Asp-Ser (IC_{50} for inhibition of aggregation was 25 µM) or fibrinogen. We have performed experiments to clarify the possible role of GPIIb-IIIa in the regulation of agonist-stimulated changes in cytosolic Ca^{2+} . For this purpose we used RGD-peptides (disintegrins) which bind with high affinity (Huang *et al.*, 1987; Williams *et al.*, 1990) to the GPIIb-IIIa receptor and compete with fibrinogen for binding, via a conformationally active Arg-Gly-Asp (RGD) sequence (Ruoslahti & Pierschbacher, 1987; Reed *et al.*, 1988). These agents are preferable to either Gly-Arg-Gly-Asp-Ser or fibrinogen, since they have a much higher affinity for the fibrinogen receptor (IC_{50} = 35–250 nM). As such, they are excellent probes with which to examine the effects of relevant receptor-ligand interactions, particularly since they inhibit, rather than promote, platelet aggregation. Fibrinogen, when used at high concentrations, produces significant aggregation which makes quantitative interpretation of fluorescence measurements impossible. In the experiments (results not shown) that we did perform in the presence of fibrinogen (400 µg/ml), we did not notice a significant effect of fibrinogen on ADP-stimulated intracellular Ca^{2+} changes, but observed a tendency for aggregation to cause an underestimation of the fluorescence measurement. Furthermore, the GPIIb-IIIa receptor is only available to bind fibrinogen after platelet activation.

The disintegrins albolabrin and elegantin inhibit platelet aggregation (Fig. 1) in platelet-rich plasma with IC_{50} values of 210 nM and 130 nM respectively (Williams *et al.*, 1989). Yet, as we have shown here, these peptides have no significant effect on Ca^{2+} influx into platelets in the presence of either nominal or 1 mM extracellular Ca^{2+} . In addition, they had no effect on either ADP- or thrombin-induced influx of Mn^{2+} , as measured by quenching of fura-2 fluorescence. In other studies not shown here, we also looked at two other agents, echistatin (IC_{50} for inhibition of aggregation 35 nM; Gan *et al.*, 1988) and Arg-Gly-Asp-Ser, and found no effect on intracellular Ca^{2+} mobilization. While this paper was in preparation, Rybak & Renzulli (1989) published data showing that with fura-2-loaded thrombin-stimulated platelets, Arg-Gly-Asp-Ser (25–125 μ M) had no effect on thrombin-induced Ca^{2+} increase. However, an analogue (His-12-Val), of the fibrinogen γ -chain dodecapeptide inhibited 'a minor component of transmembrane Ca^{2+} influx' into stimulated platelets. We, however, found no effect of the native fibrinogen γ -chain dodecapeptide ligand (100 μ M) on thrombin-induced intracellular Ca^{2+} mobilization. The reasons for this discrepancy are unclear, but may be due to the use of an analogue rather than the native peptide.

Whereas the monoclonal antibody M148, directed towards the GPIIb-IIIa complex, was found to be a good inhibitor of platelet aggregation, we failed to detect any effect of this antibody on either Ca^{2+} mobilization or Mn^{2+} influx. Although this result is in contrast with the original observations of Powling & Hardisty (1985), it is consistent with their observation that the antibody had no effect on the platelets of patients lacking the GPIIb-IIIa complex. The contradictory results of other authors with thrombasthenic platelets may be due to additional membrane defects, which have not yet been detected. The present result is also consistent with a later report by Hardisty & Powling (1989) that the purified Fab fragment of monoclonal antibody M148 had no effect on Ca^{2+} influx compared with M148 in ascitic fluid. Our conclusion is that some component of the ascites fluid other than M148 might be responsible for effects on Ca^{2+} influx. A non-specific effect of the ascites fluid may also explain the results obtained by Rybak *et al.* (1988) with the same antibody M148 and GPIIb-IIIa incorporated into liposomes, which suggested that the GPIIb-IIIa complex facilitated passive Ca^{2+} flux into the liposome.

We have also examined the effect of disintegrins on the major inhibitory second messenger pathway in platelets mediated through cyclic AMP. Several molecular mechanisms have been proposed for the role of cyclic AMP in inhibition of platelet activation, including phosphorylation and the stimulation of an ATP-dependent Ca^{2+} uptake system, removing Ca^{2+} from the cytosol and from its role in platelet activation (Feinstein *et al.*, 1983). Cyclic AMP-stimulated Ca^{2+} uptake has been demonstrated in isolated membrane vesicles (Kaser-Glanzmann *et al.*, 1979). We show here that disintegrins have no effect on platelet cyclic AMP metabolism; they do not stimulate or inhibit cyclic AMP formation in either the absence or the presence of phosphodiesterase inhibitors (Fig. 5). These findings support the view that fibrinogen binding to the activated GPIIb-IIIa complex during platelet aggregation represents a passive event, providing a surface for cross-linking of platelets through fibrinogen molecules.

The primary structures of glycoproteins IIb and IIIa are now known. Hydropathicity analysis of the sequences indicates that each polypeptide has a single membrane-spanning domain (Fitzgerald *et al.*, 1987). This is in sharp contrast with similar analysis of the primary sequence of known ion channels which appear to contain at least six and as many as 24 membrane-spanning domains (Catterall, 1989). Rybak *et al.* (1988) have

demonstrated that GPIIb-IIIa can in some way enhance passive Ca^{2+} entry into an artificial liposome. It is not clear that this effect is related to a Ca^{2+} channel, since it does not have the properties of the putative receptor-operated channels of platelets, which requires occupancy of a receptor to allow Ca^{2+} influx (Rink, 1988). In addition, the studies of Zschauer *et al.* (1988) indicated that the platelet membrane has extremely low conductance to Ca^{2+} , suggesting that a passive channel is not present. Our own data agree with this view since we failed to detect an effect of blocking of the GPIIb-IIIa receptor with either disintegrins or monoclonal antibody M148 on agonist-stimulated Ca^{2+} influx. We have also ruled out the possibility that fibrinogen binding may be indirectly modulating Ca^{2+} levels by affecting intracellular cyclic AMP.

In the light of our findings, we suggest that the disintegrins may serve as useful agents for studies of platelet activation pathways *in vitro*, in which it may be necessary to prevent unwanted fibrinogen-mediated aggregation.

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