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Inhibition of platelet function with synthetic peptides designed to be high-affinity antagonists of fibrinogen binding to platelets

(platelet receptors/platelet aggregation/thrombosis)

ZAVERIO M. RUGGERI*, RICHARD A. HOUGHTEN, SUSAN R. RUSSELL, AND THEODORE S. ZIMMERMAN

Departments of Basic and Clinical Research and Molecular Biology, Scripps Clinic and Research Foundation, La Jolla, CA 92037

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ABSTRACT **We have constructed synthetic peptides modeled on the sequences of (*i*) Arg-Gly-Asp, present in fibrinogen, fibronectin, and von Willebrand factor, and of (*ii*) the fibrinogen γ chain (γ 400–411) His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val. The concentration of each peptide that inhibits 50% of ^{125}I-labeled fibrinogen binding to thrombin-stimulated platelets (IC_{50}) was then determined. The IC_{50} for (γ 400–411) was 48–180 μM at a fibrinogen concentration of 60 μg/ml. A substitution of arginine for alanine at position 9 decreased the IC_{50} to 14.5 μM. Arginine substitutions for all other residues on the amino-terminal side of the peptide Arg_9-Gly-Asp-Val resulted in an IC_{50} of 0.4–0.8 μM, and the IC_{50} of the peptide Arg_{13}-Gly-Asp-Val was 0.2–0.3 μM. This contrasts with an IC_{50} of 200 μM for Arg_5-Gly-Asp-Val-Arg_4 and an IC_{50} >1 mM for the peptide Arg_{12}. The inhibitory effect resulted primarily in a decreased affinity of fibrinogen binding to platelets, although the number of available binding sites had also decreased. Binding was completely inhibited. At concentrations between 10 and 18 μM, Arg_9-Gly-Asp-Val blocked all ADP-induced aggregation in citrated platelet-rich plasma. The peptide Tyr-His-His-Lys-Arg-Lys-Arg-Lys-Gln-Arg-Gly-Asp-Val was labeled with ^{125}I to quantitate its binding to thrombin-stimulated platelets; at saturation, 59,990 molecules were bound per cell ($K_d = 3.8 \times 10^{-7}$ M). These modified synthetic peptides bind to platelets with the same affinity as does intact fibrinogen and inhibit platelet function. The increased affinity of these modified peptides is >20-fold that of peptides comprised of only native sequences and is a prerequisite for the potential antithrombotic use of these agents.**

The interaction of fibrinogen with specific membrane receptors is essential for normal platelet function (1–3), as demonstrated by prolongation of bleeding time in patients with afibrinogenemia (4). The fibrinogen binding sites involved in platelet aggregation have been located on the platelet membrane glycoprotein (GP) complex IIb/IIIa (5, 6). Evidence has recently been obtained that von Willebrand factor and fibronectin also bind to the GPIIb/IIIa complex on stimulated platelets (7, 8) and may participate in promoting platelet–platelet interactions (9, 10). In addition, von Willebrand factor binds to the membrane receptor GPIb and mediates both platelet adhesion and spreading at sites of vascular injury, particularly under conditions of high shear rate (11, 12).

Three distinct amino acid sequences seem to be involved in the platelet-attachment function of fibrinogen. They are: Gly-Pro-Arg (13), which corresponds to the amino terminus of the α chain of fibrin; His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (14, 15), the carboxyl terminus of the fibrinogen γ chain (γ 400–411); and Arg-Gly-Asp (16, 17), which recurs twice in the fibrinogen α chain. This latter sequence, also found in von Willebrand factor and fibronectin (18, 19), probably mediates the binding of these two proteins to the platelet GPIIb/IIIa receptor (17, 20).

Small synthetic peptides that have an amino acid sequence identical to that described in the fibrinogen molecule retain an ability to block fibrinogen–platelet interaction (13–17). The affinity of these synthetic peptides for platelets, however, is less than one-tenth that of native fibrinogen. We describe a series of synthetic peptides, designed in lengths to 16 residues, that contain the sequence Arg-Gly-Asp-Val and have 20-fold greater affinity for platelets than previously described molecules. These synthetic peptides inhibit fibrinogen binding to platelets at concentrations similar to the dissociation constant (K_d) of intact fibrinogen.

MATERIALS AND METHODS

Peptide Synthesis and Characterization. All peptides were synthesized by the method of simultaneous multiple peptide synthesis (SMPS), which has been described in detail (21). These peptides were 60–80% pure, as judged by reverse-phase HPLC (Perkin–Elmer) performed with a 1 × 25 cm Vydac C-18 column (TP silica; pore diameter, 300 Å) with a 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid; the peptides were not further purified for the screening experiments used to evaluate inhibition of fibrinogen binding to platelets. Peptides with high inhibitory activities were purified to homogeneity, using the chromatographic methods indicated above, in order to evaluate the inhibitory effects of these purified peptides on fibrinogen binding to platelets. Contaminants of the preparations were molecules that lacked random residues due to incomplete coupling during synthesis. The tyrosine-containing peptide that was later radioiodinated to directly measure binding to platelets was also purified to homogeneity. The amino acid composition of each preparation was determined with an automated amino acid analyzer (LKB) after 24-hr hydrolysis in 6 M HCl at 110°C and sequences were consistent with those predicted. Peptides were stored in the lyophilized state and dissolved in distilled water immediately before use.

Purification of Fibrinogen. Fibrinogen was purified from blood collected in acid/citrate/dextrose anticoagulant containing 0.1 M (final concentration) ε-aminocaproic acid, using the glycine precipitation method of Kazal *et al.* (22). Finally, traces of contaminating von Willebrand factor were removed by gel filtration through an 80 × 5 cm Sepharose 4B-CL

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Abbreviations: GP, platelet membrane glycoprotein; K_d, dissociation constant; IC_{50}, peptide concentration that inhibits 50% of fibrinogen binding to thrombin-stimulated platelets; γ 400–411, the fibrinogen γ chain (His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val).

*To whom reprint requests should be addressed at: Department of Basic and Clinical Research (BCR8), Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037.

column (Pharmacia). In the purified fibrinogen preparation, 95% of the protein was clotted by addition of α-thrombin.

Radiolabeling of Molecules. This was achieved using carrier-free $Na^{125}I$ (Amersham) and Iodo-Gen (Pierce), following the procedure described by Fraker and Speck (23).

Binding Studies. Platelets were washed free of plasma constituents by the albumin density-gradient technique of Walsh *et al.* (24), with modifications previously described (25). In each experimental mixture, platelets in modified Tyrode's buffer (7) were stimulated with human α-thrombin (the generous gift of John W. Fenton II) at 22–25°C for 10 min (3.125×10^{11} platelets per liter and thrombin at 0.1 NIH units/ml). Hirudin (Sigma) was then added at a 25-fold excess (unit/unit) for 5 min before addition of the radiolabeled ligand and any competing ligand. After these additions, the final platelet count in the mixture was 1×10^{11}/liter. After incubation for an additional 30 min at 22-25°C, bound and free ligand were separated by centrifuging 50 μl of the mixture through 300 μl of 20% sucrose at 12,000 × *g* for 4 min (7). The platelet pellet was separated from the rest of the mixture to determine platelet-bound radioactivity. Nonspecific binding was measured in mixtures containing an excess of unlabeled ligand. When binding curves were analyzed by Scatchard analysis (26), nonspecific binding was derived as a fitted parameter from the binding isotherm by means of a computerized program that has been described previously (27). To determine the concentration of each inhibitory peptide necessary to inhibit 50% of fibrinogen binding to thrombin-stimulated platelets (IC_{50}), each compound was tested at six or more concentrations with ^{125}I-labeled fibrinogen held at 0.176 μmol/liter (60 μg/ml). The IC_{50} was derived by plotting residual fibrinogen binding against the logarithm of peptide concentration.

Aggregation Studies. Blood was drawn into trisodium citrate (final concentration of 11 mM) and platelet-rich plasma was prepared as described previously (7). Aggregation studies were completed within 1 hr from blood collection. Reaction mixtures were prepared in siliconized glass cuvettes, and the experiments were performed in the aggregometer (Chrono-Log, Havertown, PA), at 37°C, with constant stirring of the platelet suspension at 1200 rpm; final platelet count was between $2.5–3 \times 10^{11}$/liter. Stimuli included ADP (Sigma), epinephrine (Parke, Davis), collagen (equine collagen fibrils; Hormon-Chemie, Munich, F.R.G.), and human α-thrombin. Aggregation was quantitated by monitoring increase in light transmittance through the stirred platelet suspension.

Release Studies. The release of serotonin from thrombin-stimulated platelets was measured after labeling the platelets with 5-hydroxy[2-^{14}C]tryptamine creatinine sulfate (Amersham) and using the previously described method (7).

RESULTS

Several analogs of the dodecapeptide His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (residues 400–411 of the fibrinogen γ chain) were synthesized and tested to determine the IC_{50} (Fig. 1). Each synthesized peptide had a single amino acid substitution as compared with the native peptide. The IC_{50} value of the parent peptide, synthesized on four occasions and tested in eight experiments, varied from 48 to 180 μM. The analog containing a substitution of arginine for alanine at position 9 had the lowest IC_{50} in the group (14.5 μM). This particular peptide contains the sequence Arg-Gly-Asp, one present in the active tetrapeptide Arg-Gly-Asp-Ser that represents the cell attachment site of fibronectin (19). In parallel experiments, the IC_{50} for Arg-Gly-Asp-Ser was found to be 48 μM. The arginine residue at position 9 proved critical for the increased affinity of the modified peptide; a conservative exchange of lysine for arginine at that position diminished the molecule's activity (Fig. 1). Single substitutions at positions Leu-3, Lys-7, Gly-10, and Asp-11 increased the IC_{50} over that of the unmodified sequence. Yet, several amino acids could replace Gly-4 and Gly-5 without reducing the inhibitory activity of the dodecapeptide (Fig. 1).

We then studied the effect of multiple amino acid substitutions in the dodecapeptide His-His-Leu-Gly-Gly-Ala-Lys-Gln-Arg-Gly-Asp-Val (Table 1). When arginine or lysine was substituted at positions 1–8 the resulting molecules had lower IC_{50} values. The two peptides with the greatest inhibitory

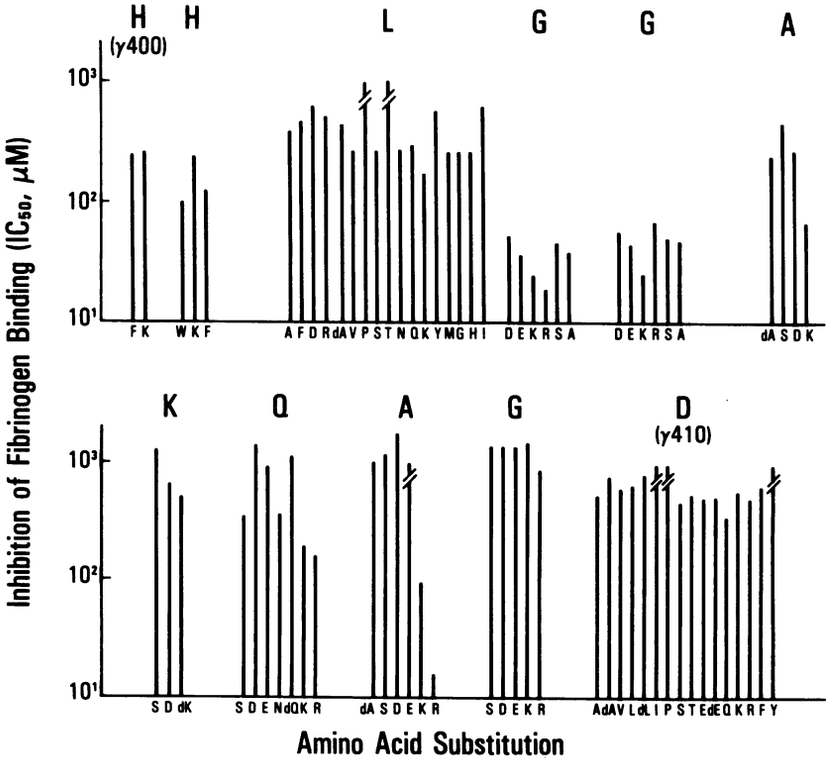

FIG. 1. Effect of single amino acid substitutions on the inhibitory effect of the dodecapeptide γ 400–411 on fibrinogen binding to thrombin-stimulated platelets. Standard one-letter abbreviation identifies all amino acids; the prefix d denotes the D-amino acid configuration in the modified analog (28). The naturally occurring residue is indicated for each position above the group of amino acids used in the modified analogs. The vertical lines on the graph indicate the concentration of each modified peptide (in μmol/liter) necessary to inhibit 50% of the binding (IC_{50}) of fibrinogen present at a concentration of 60 μg/ml (0.176 μmol/liter). The IC_{50} of the native peptide (γ 400–411) was between 48–180 μmol/liter. Nonspecific binding, measured in mixtures containing a 65-fold excess of unlabeled fibrinogen, never exceeded 10% of total binding and was subtracted from results to obtain specific binding.

Table 1. Effect of multiple amino acid substitutions on the IC_{50} of the peptide HHLGGAKQRGDV

Peptide	IC_{50}, μM
H H L G G A K Q R G D V	14.5
H H L G G R K Q R G D V	6.3
H H L G G K K Q R G D V	13.2
H H L G R R K Q R G D V	5.4
H H L R G R K Q R G D V	6.2
H H L R G K K Q R G D V	11.0
H H dA G G R K Q R G D V	2.8
H H L R K R K Q R G D V	4.4
H H K R K R K Q R G D V	3.3
H H K R K R K Q R G D S	6.2
H H dA R K R K Q R G D V	2.2
H H K R K R K R R G D S	3.1
H H K K K K K K R G D V	1.6
H H R R R R R R R G D V	1.3
K K K K K K K K R G D V	4.1
R R R R R R R R R G D V	0.4
K R K R K R K R R G D V	0.6
K R K R K R K R K R K R	>540

Underlined residues indicate substitutions. The prefix d denotes the D-amino acid configuration in the modified analog.

activity were $(Lys\text{-}Arg)_4$-Arg-Gly-Asp-Val and Arg_9-Gly-Asp-Val (Table 1 and Fig. 2). The inhibitory effect of the latter was the same when tested after purification to homogeneity. On the other hand, the peptides $(Lys\text{-}Arg)_6$ (Table 1) and Arg_{12} (Table 2) had no significant inhibitory activity on fibrinogen binding (IC_{50} > 540 μM).

The correlation between the number of arginine residues in peptides containing a carboxyl terminal sequence of Arg-Gly-Asp-Val and the inhibitory effect on fibrinogen binding is shown in Table 2. The number of arginine residues totaled 9–13 in analogs that had the highest affinity (lowest IC_{50}). When arginine residues were located on the carboxyl-terminal side of Arg-Gly-Asp-Val, however, that affinity was greatly decreased.

Experiments were designed to clarify the inhibitory mechanisms of the peptide Arg_9-Gly-Asp-Val. This peptide had similar effects when the calcium concentration varied from 1 to 5 mM, which suggests that the peptide does not act by decreasing divalent cations, ions necessary for fibrinogen binding to the GPIIb/IIIa complex. The peptide, moreover, did not cause dissociation of the GPIIb/IIIa complex, as demonstrated by its lack of effect on the binding of a monoclonal antibody (LJP9) that recognizes only the complex formed by GPIIb/IIIa and not the individual components (25). The peptide did not induce the release of [^{14}C]serotonin from platelets nor did it inhibit the release induced by thrombin. The binding of von Willebrand factor to platelets was blocked as effectively as the binding of fibrinogen.

Table 2. Effect of number of arginine residues on IC_{50} of different peptides

Peptide	IC_{50}, μM
R G D V	6–11
R R G D V	1.2–6
R R R G D V	2.9–3.6
R R R R G D V	1.3–4.4
R R R R R G D V	1.5–3.4
R R R R R R G D V	1.2–2.3
R R R R R R R G D V	1.0–1.5
R R R R R R R R G D V	1.1–1.3
R R R R R R R R R G D V	0.4–0.8
R R R R R R R R R R G D V	0.6–0.8
R R R R R R R R R R R G D V	0.5–0.7
R R R R R R R R R R R R G D V	0.2–0.8
R R R R R R R R R R R R R G D V	0.2–0.3
R R R R R G D V R R R R	200
R R R R R R R R R R R R	>1000

Results indicate the range of values determined in at least three separate experiments, with peptides obtained from two separate syntheses.

Arg$_9$-Gly-Asp-Val markedly decreased the affinity of fibrinogen binding to platelets (Fig. 2). The peptide, however, also caused a decrease in the number of available fibrinogen binding sites (Fig. 2). This effect on fibrinogen binding was not due to a generalized inhibitory effect on platelet function, as the inhibitory activity could be partially overcome by increasing the fibrinogen concentration.

Another peptide, Tyr-His-His-Lys-Arg-Lys-Arg-Lys-Gln-Arg-Gly-Asp-Val, was labeled with ^{125}I to determine the parameters of its platelet binding. When tested for the capacity to inhibit fibrinogen binding to platelets, this pep-

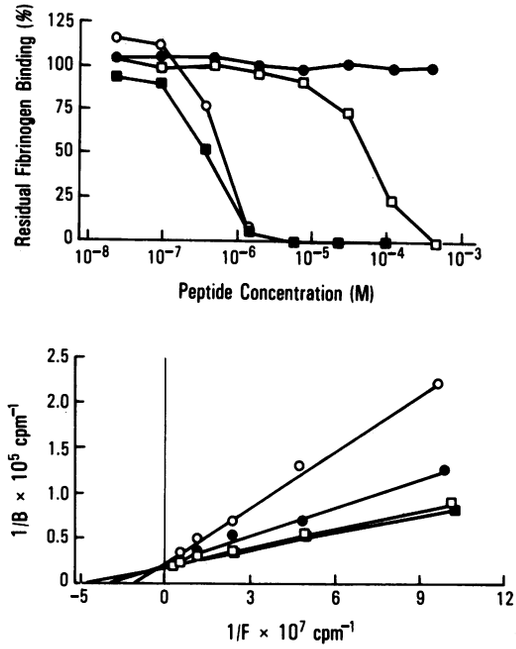

FIG. 2. Characterization of the inhibitory effect of synthetic analogs on fibrinogen binding to platelets. (*Upper*) Inhibition of fibrinogen binding to thrombin-stimulated platelets as a function of peptide concentration (■, RRRRRRRRRGDV; ○, RRRRRGDV; □, HHLGGAKQAGDV; ●, RRRRRRRRRRR). All inhibitory peptides, when used in sufficient concentration, completely blocked fibrinogen binding. In these experiments fibrinogen was present at a constant concentration of 0.176 μmol/liter (60 μg/ml). One hundred percent binding corresponded to 20,000–40,000 molecules of fibrinogen per platelet. (*Lower*) Binding of fibrinogen at different concentrations to thrombin-stimulated platelets in the presence of various concentrations of RRRRRRRRRGDV. (○, 1 μmol/liter; ●, 0.5 μmol/liter; □, 0.25 μmol/liter; ■ = 0). A double reciprocal plot of [1/bound fibrinogen (B) versus 1/free fibrinogen (F)] is shown, which demonstrates that presence of the peptide decreases affinity of binding (x intercept) with only a modest effect on the total number of available binding sites (y intercept). These experiments were repeated three times, and the binding data were also analyzed by Scatchard plot (26). All curves could be fit to a linear one-site model, using the computer-assisted Ligand program (27). In the absence of competing peptide, the K_d of fibrinogen binding to thrombin-stimulated platelets was 1.5–5.4 × 10^{-7} M. At the highest dose of the peptide RRRRRRRRRGDV tested (1 μM), the K_d was 2–4 times greater than in the absence of competing peptide in all three experiments. The number of binding sites was either unchanged (one experiment) or decreased 35–59% (two experiments).

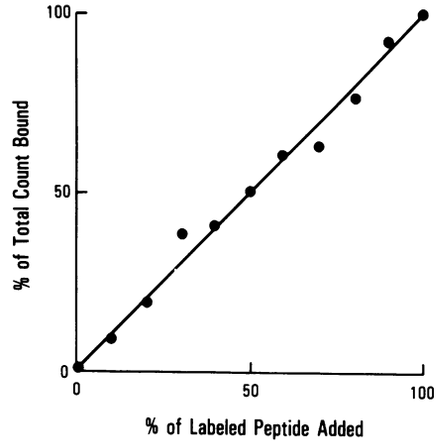

FIG. 3. Relative binding of labeled and unlabeled peptide YHHKRKRKQRGDV to thrombin-stimulated platelets. The total peptide concentration was kept constant in the mixture (1.7 μmol/liter), but the proportion of labeled species was varied from 0 to 100%. A plot of % of count bound versus % of labeled peptide added gave a linear correlation ($y = 2.16x + 0.98$). The slope of the curve (0.98) was close to the value of 1, expected when labeled and unlabeled species bind with the same affinity. The specific activity of the labeled peptide was 19.8×10^3 Ci/mol (733 TBq/mol).

tide, purified to homogeneity, reached its IC_{50} at 0.5 μM. In a preliminary experiment, we had demonstrated that the labeled and unlabeled species of the Tyr-containing peptide had similar affinities for platelets (Fig. 3). The labeled peptide bound to a single class of sites on thrombin-stimulated platelets and, at saturation, 59,990 molecules per platelet were bound ($K_d = 3.8 \times 10^{-7}$ M) (Fig. 4). The binding to unstimulated platelets was <20% of the binding to stimulated

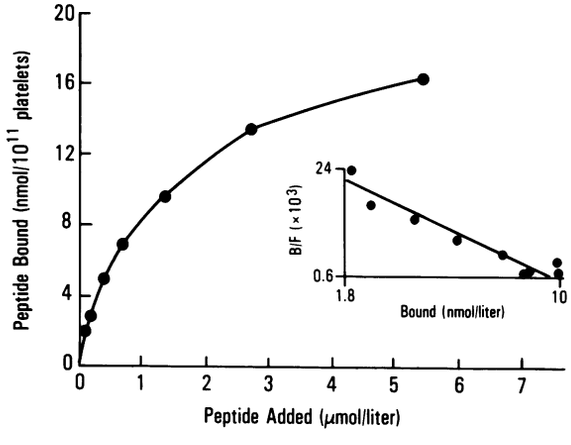

FIG. 4. Saturation binding of the labeled peptide, YHHKRKRKQRDGV, to thrombin-stimulated platelets. The labeled peptide was added to thrombin-stimulated platelets at nine concentrations between 0.09 and 16 μmol/liter (the two higher values are not shown on the graph to allow a better representation of the lower values). The curve shows the total amount of peptide bound as a function of the peptide concentration added and clearly demonstrates the tendency to saturation. Binding of the labeled peptide was decreased by at least 70% when a 50-fold excess of unlabeled peptide was present in the mixture. Binding isotherms were analyzed with a computer-assisted program (27), and nonspecific (nonsaturable) binding was calculated as a fitted parameter. In preliminary experiments we determined that 80% of the bound peptide could be displaced in 5 min after addition of a 50-fold excess of unlabeled peptide. (*Inset*) A Scatchard-type plot [bound/free (B/F) versus bound] representing the calculated specific binding of all the experimental points, including the two not shown in the curve. The following results were obtained: number of binding sites = 59,990 per platelet; $K_d = 3.8 \times 10^{-7}$ M; nonspecific (nonsaturable) binding = 1.4×10^{-3}.

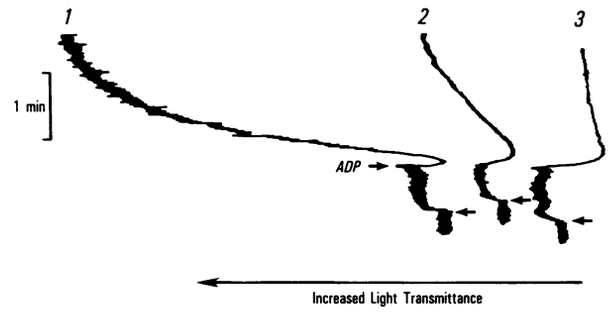

FIG. 5. Effect of peptide analogs on platelet aggregation. Aggregation of platelet-rich plasma was induced by ADP added to a final concentration of 2 μM (upper arrow). Aggregation was recorded as increase in light transmittance through the stirred platelet suspension. Curve 1: control experiment in which buffer was added to the mixture (lower arrow) instead of the peptide tested. Curve 2: effect of RRRRRRRRRGDV at a final concentration of 10 μM. Curve 3: effect of the same peptide at 18 μM. Note that the peptide does not inhibit platelet shape change, as shown by the decrease in light transmittance following addition of ADP. The effect of the peptide was immediate and independent of preincubation with platelets.

platelets. In experiments to be reported elsewhere, we also determined that the peptide does not bind to thrombasthenic platelets.

The effect of the peptide Arg_9-Gly-Asp-Val on platelet aggregation was also evaluated. Complete inhibition of the aggregation induced by ADP in normal platelet-rich plasma could be achieved at peptide concentrations between 10 and 18 μM (Fig. 5); the average fibrinogen concentration in plasma is 7–9 μM. That ADP-induced change in platelet shape was not inhibited by the peptide lends additional support to the contention that this molecule does not act as a generalized inhibitor of platelet function. Moreover, the inhibitory effect of the peptide on platelet aggregation could be partially overcome by addition of purified fibrinogen to the platelet-rich plasma. Thrombin-induced aggregation, as well as aggregation induced by collagen and epinephrine, was also completely blocked by the peptide at a concentration of 20 μM, an effect that did not require preincubation with the platelets.

DISCUSSION

The synthetic peptides here described are modeled on short amino acid sequences known to occur in adhesive glycoproteins that bind to platelet membrane receptors on the GPIIb/IIIa complex. Modified peptides can be synthesized that bind to platelets with a K_d in the order of 10^{-7} M, which is similar to that of native fibrinogen (2, 3). At concentrations of 10–20 μM these modified peptides completely inhibit ADP-induced aggregation in platelet-rich plasma, in which fibrinogen concentration is ≈8 μM.

Some of the discrepancies in the reported inhibitory potencies of a given peptide can be explained by the different fibrinogen concentrations used in the binding assays. In this study we have used identical assay conditions to compare the IC_{50} values of our designed molecules and previously described peptides that contain the sequence of γ 400–411 or Arg-Gly-Asp. The IC_{50} values we report for those earlier described peptides agree with literature-reported values (14–17, 20). This is good evidence that the peptides recently designed in our laboratory have at least 20-fold the inhibitory potency on fibrinogen-platelet interaction as previously described molecules.

Moreover, the peptides here described have a higher affinity for platelets than those reported, as shown by direct binding studies. Kloczewiak *et al.* (29) reported that a molecule related to fibrinogen γ 400–411 bound to platelets

with a K_d of 3×10^{-4} M, whereas Plow *et al.* (17) described a peptide containing the sequence Arg-Gly-Asp that had a K_d of 9×10^{-6} M. One of our modified peptides with a low, though not the lowest, IC_{50} bound to platelets with a K_d of 3.8×10^{-7} M.

Structural requirements for the high affinity of these modified peptides include the sequence Arg-Gly-Asp at the carboxyl terminus of the molecule; this sequence appears to be necessary for receptor specificity (17, 19). In addition, a basic, hydrophilic amino terminal sequence consisting preferably of 8–12 arginine residues or arginine alternating with lysine residues, confers the increased affinity. Yet, arginine residues at the carboxyl terminus of a peptide containing Arg-Gly-Asp-Val decrease the molecule's activity to 1/1000th that of a peptide containing the same number of arginine residues but a carboxyl-terminal sequence of Arg-Gly-Asp-Val. This demonstrates that sequence specificity as well as basic-residue content are essential for the high affinity of the modified peptides.

The inhibitory peptides had a profound effect on the K_d of the fibrinogen–platelet interaction and a less pronounced effect on the number of available binding sites. The inhibitory effect could generally be overcome by increasing fibrinogen concentration. The inhibitory effect of the peptides on the aggregation of platelet-rich plasma, thus, could be partially overcome by the addition of exogenous fibrinogen. We also found that the number of sites on stimulated platelets to which the peptides bound was close to the accepted number of fibrinogen receptors (2, 3). It is probable, therefore, that these peptides bind to a platelet membrane structure that closely resembles the fibrinogen receptor.

The existence of amino acid sequences that mediate platelet interaction with adhesive proteins suggests that peptides bearing such sequences would have a potential for modulating platelet thrombus formation. For this approach to be practical, agents must be developed that retain the specificity of peptides containing native amino acid sequences but that have high affinity for *in vivo* use. The synthetic peptides described in this report, 16 (or fewer) amino acid residues long and comparable to intact fibrinogen in platelet receptor specificity and affinity, illustrate the feasibility of tailoring molecules with both features.

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