A novel approach to the design of potent bioactive peptides by incorporation of proline brackets: antiplatelet effects of Arg-Gly-Asp peptides

R. Manjunatha Kini*, Herbert J. Evans

Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0614, USA

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Abstract Enhancing the potency of peptides is a critical and important step in the development of peptide drugs. We have proposed that proline residues flanking protein–protein interaction sites perform a structural role in enhancing their interaction [R.M. Kini and H.J. Evans, *Biochem. Biophys. Res. Commun.* 212 (1995) 1115–1124]. To test this theory, we incorporated proline residues on either or both sides of the interaction site of an antiplatelet peptide, IARGDMNA and determined the inhibitory potency of the peptides in whole blood aggregation. Inclusion of one proline residue, on either the amino or carboxy terminal side of the interaction site, enhances the antiplatelet activity to approximately the same extent (1.5- to 2.5-fold). Incorporation of proline residues on both sides enhances the activity by 7- to 13-fold. This enhancement of the biological activity of the peptide is probably due to a reduction in the number of possible conformations of the peptide, without introducing the rigidity that would accompany cyclization. Incorporation of proline brackets thus provides a novel approach to the design and development of more potent peptide drugs and ligands.

Key words: Platelet aggregation; Design (de novo); Bioactive peptide; Protein–protein interaction; Interaction site; Molecular recognition

1. Introduction

Several bioactive peptide drugs interfere in protein–protein interactions. The potency of bioactive peptides is directly proportional to their ability to interact with their target proteins. Structural features that enhance the protein–protein interactions can therefore contribute to the design and development of potent bioactive peptides. Recently, by a survey of over 1600 protein–protein interaction sites, we recognized that proline is the most common amino acid residue found in the flanking segments of interaction sites [1]. We proposed that proline residues prevent the extension of neighboring secondary structures and thus protect the conformation and integrity of interaction sites. These proline residues also help in the presentation of interaction sites [1]. If this hypothetical structural role is true, it is logical to propose that the incorporation of proline residues around a bioactive peptide might enhance its potency significantly. In this report, we therefore examined the effect of incorporation of proline residues on the antiplatelet potency of peptides containing the Arg-Gly-Asp (RGD) sequence. We chose to study the effect of incorporation of proline brackets in an antiplatelet peptide for the following reasons: (a) development of antiplatelet drugs is important in the prevention/treatment of atherosclerosis, myocardial infarction, stroke and cancer [2–4]; (b) the proteins involved in platelet aggregation and other adhesive interactions are structurally and immunologically related [5]; and (c) platelet aggregation, a specialized adhesive reaction, is easy to monitor by facile methods. Platelet aggregation is mediated via interaction between platelet integrin, the glycoprotein IIb–IIIa complex, and adhesive protein ligands, such as fibrinogen, fibronectin and von Willebrand factor [6,7]

The Arg-Gly-Asp (RGD) tripeptide is the most common molecular recognition site implicated in several of these interactions [8–10]. Small peptides containing the RGD sequence inhibit adhesive reactions including platelet aggregation. We selected IARGDMNA as a typical RGD-containing peptide, since related sequences are found in disintegrins, the most potent inhibitors of platelet aggregation, from snake venoms [11–13]. Proline residues were substituted on either side or both sides of the RGDM sequence. All four peptides, IARGDMNA, IARGDMPA, IPRGDMNA and IPRGDMPA, were synthesized by solid phase peptides synthesis. The effect of incorporation of proline residues on the antiplatelet effect was studied.

2. Materials and methods

2.1. Materials

t-Butyloxycarbonyl (*t*-BOC) amino acids and trifluoroacetic acid were obtained from Advanced Chemtech, and other reagents and solvents were obtained from Fisher Scientific. Collagen and ADP used to initiate platelet aggregation were from Chrono-Log Corp.

2.2. Peptide synthesis

We synthesized peptides using *t*-BOC chemistry on Merrifield resin [14], using a Milligen/Biosearch Model 9600 peptide synthesizer. After extraction, the peptides were purified by reverse phase HPLC to more than 95% purity, with yields between 80 and 90%. Amino acid analyses were performed after 24 h hydrolysis of the peptides in 6 N HCl at 110°C. The amino acid compositions were similar to those expected from the sequences of the peptides within experimental errors. The structures were also confirmed by determining the mass by fast atom bombardment mass spectra on a Finnegan TSQ 70 mass spectrometer. The masses of IARGDMNA, IPRGDMNA, IARGDMPA and IPRGDMPA as determined by mass spectrometry were 847 (846.96 calculated), 873 (873.00), 831 (829.97) and 855 (856.01), respectively.

2.3. Platelet aggregations

Blood was drawn and anticoagulated with 0.11 M trisodium citrate (1:9, v/v), from healthy volunteers who had not taken any medications or alcohol in the previous week. The citrated blood was diluted 1:1 with phosphate-buffered saline, and 1 ml of the diluted blood was incubated

*Corresponding author. *Present address:* Bioscience Centre, Faculty of Science, National University of Singapore, Lower Kent Ridge Road, Singapore 0511, Republic of Singapore. Fax: (65) 779-2486.

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with various amounts of peptide or buffer at 37°C for 2 min. Platelet aggregation was initiated by the addition of 2 μl collagen (1 mg/ml) or ADP (10 μM final concentration). The aggregation was measured as the increase in electrical impedance in a whole blood aggregometer [15]. The percent inhibition was calculated by comparing the impedance of the aggregation curves 5 min after the addition of collagen or ADP, taking the impedance of the control curves as 100% aggregation.

3. Results and discussion

We examined the effect of the peptides, IARGDMNA, IPRGDMNA, IARGDMPA and IPRGDMPA, on whole blood aggregation. This assay system more closely mimics physiological conditions than the aggregation of washed or gel-filtered platelets. Moreover, there is a direct correlation between inhibition of binding of fibrinogen to glycoprotein IIb–IIIa complex and platelet aggregation [16]. All four peptides inhibited platelet aggregation (Fig. 1). To compare inhibitory potencies, we determined the dose–response relationships for all the peptides (Fig. 2). We estimated IC_{50} values (concentration of the peptide that inhibits platelet aggregation by 50%) from the dose–response curves and the fold increase in the inhibitory potencies (Table 1). As expected according to our proposal, the inhibitory potencies of the peptides were IPRGDMPA > IPRGDMNA = IARGDMPA > IARGDMNA. The potency of IARGDMNA is comparable with that of the RGDS peptide [17]. Incorporation of a proline residue on either side of RGDM enhances the potency to about the same extent. Inclusion of proline residues on both sides enhanced the antiplatelet effect of the RGD peptide by 7- to 13-fold. These results show that proline brackets enhance the biological activity of the antiplatelet peptide by amplifying its ability to interfere in the interaction between fibrinogen and its receptor [16].

In proteins proline brackets protect and present protein–protein interaction sites [1]. In small peptides containing the minimum molecular recognition sites, since there are no adjacent secondary structures, proline brackets probably help in the

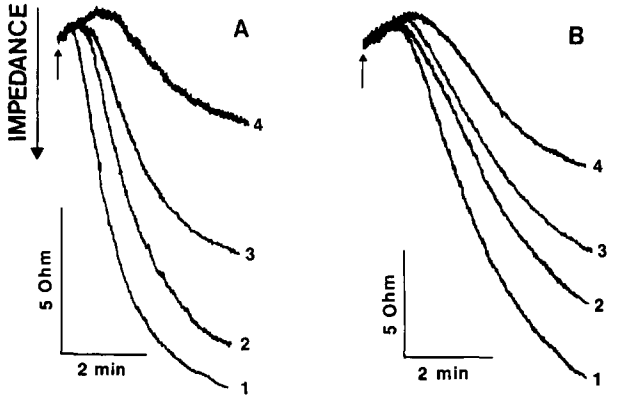

Fig. 1. Effect of various peptides on platelet aggregation. Panel A: ADP-induced aggregation . Curves 2, 3 and 4 show the effect of 15 μM IARGDMNA, 12.5 μM IARGDMPA and 3.5 μM IPRGDMPA, respectively, on control aggregation (curve 1). Panel B: collagen-induced aggregation. Curves 2, 3 and 4 show the effect of 50 μM IARGDMNA, 20 μM IARGDMPA and 10 μM IPRGDMPA, respectively, on control aggregation (curve 1). The arrow indicates the addition of either ADP (10 μM) or collagen (2 μg) to initiate aggregation. The increase in the impedance of the aggregation induced by collagen is larger than that induced by ADP [20]. Typical of at least two experiments. Similar relative inhibitory potencies for the peptides were observed in several independent experiments performed on separate days.

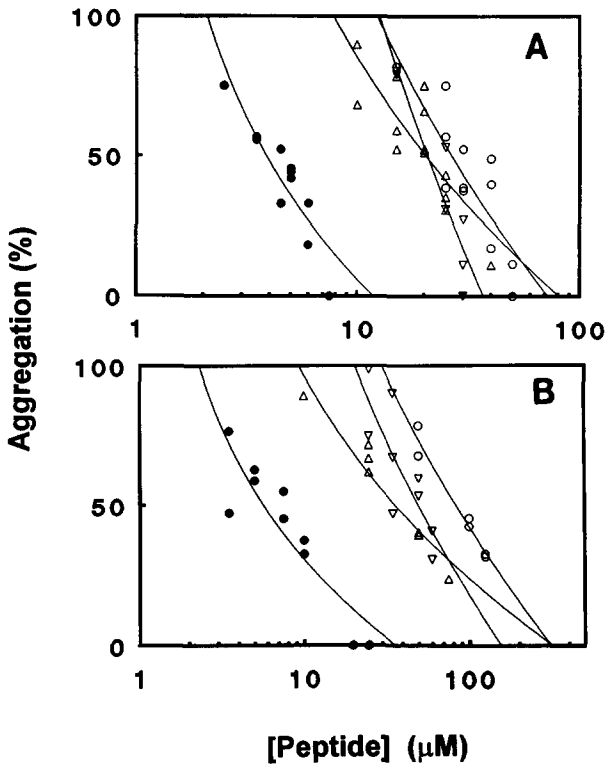

Fig. 2. Dose–response curves of various peptides on platelet aggregation. Various amounts of individual peptides were incubated with diluted whole blood for two min at 37°C before the addition of ADP (panel A) or collagen (panel B). ●, IPRGDMPA; ○, IARGDMNA; △, IARGDMPA; and ▽, IPRGDMNA. Dose–response curves shown here are obtained from the blood of donor 1. Similar curves were also obtained using the blood of donor 2. Similar relative potencies were observed when experiments were performed on at least three separate days. The collagen-induced aggregation is comparatively more resistant to inhibition.

presentation of the site. In these peptides, the lack of secondary structure-stabilizing features, such as reinforcing hydrogen bonds, prevent definite secondary structures, and the peptides occur in many relatively unstable conformations. Since proline limits the flexibility around the α-carbon atoms [1], the number of possible conformations would be drastically reduced. Thus, the peptide with the proper conformation would be much more probable, resulting in greater potency of peptides with proline brackets. Further studies are required to clarify the contribution of proline brackets to the secondary structures of these peptides under various experimental conditions.

The potency of peptides can also be enhanced by cyclization [18,19] which also reduces the number of conformations. Unlike the case with proline brackets, the conformational restraints in cyclization are due to a covalent cross-link, which may reduce the flexibility too much and even result in strain at the interaction site and loss of its function. Proline brackets, however, introduce no undue strain along the backbone, and thus allow the flexibility of the interaction site. An increase in potency of 10- to 15-fold is highly significant and can be critical in development of bioactive peptides. For example, Barker et al. synthesized more than 80 cyclic RGD peptide analogs and obtained a potent platelet aggregation inhibitor, G-4120, with an IC_{50} of 0.15 μM [18]. The peptide with complete proline brackets, IPRGDMPA, inhibits platelet aggregation with an

Table 1
Effect of proline brackets on potency of inhibition of platelet aggregation

Peptide	Donor 1 (female) IC_{50} (μM)	Fold	Donor 2 (male) IC_{50} (μM)	Fold
Collagen-induced aggregation				
IARGDMNA	84.5	–	67.3	–
IPRGDMNA	48.8	1.73	27.6	2.44
IARGDMPA	37.5	2.25	27.6	2.44
IPRGDMPA	6.4	13.10	8.4	8.01
RGDS	57.8	–	32.3	–
ADP-induced aggregation				
IARGDMNA	27.3	–	22.5	–
IPRGDMNA	21.5	1.27	18.9	1.19
IARGDMPA	21.0	1.30	16.7	1.34
IPRGDMPA	4.03	6.77	2.2	10.27
RGDS	29.9	–	13.75	–

IC_{50} values were calculated from two separate individuals. Similar dose–responses and inhibitory potencies were obtained by repeating these studies. The values are within 20% confidence limits.
*The inhibitory potency of each peptide was compared with that of IARGDMNA to obtain the fold increase in its potency.

IC_{50} of 2.2–4.0 μM. Simply by including proline brackets, we obtained a potent antiplatelet peptide that should provide a strong starting point for the development of more potent antithrombotic peptides. Thus this novel approach of incorporation of proline brackets provides a viable alternative to cyclization in the design and development of potent peptide drugs and ligands, a critical step in their development as drugs. These results provide strong support for our hypothesis of the importance of proline brackets [1].

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