Mapping the interaction of bradykinin 1–5 with the exodomain of human protease activated receptor 4

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Abstract The angiotensin converting enzyme breakdown product of bradykinin, bradykinin 1–5 (RPPGF), inhibits thrombin-induced human or mouse platelet aggregation. RPPGF binds to the exodomain of human protease-activated receptor 1 (PAR1). Studies determined if RPPGF also binds to the exodomain of human PAR4. RPPGF binds to a peptide of the thrombin cleavage site on PAR4. Recombinant wild-type and mutated exodomain of human PAR4 was prepared. The N-terminal arginine on RPPGF binds to the P2 position or proline[46] on PAR4 to block thrombin cleavage. These data indicate that RPPGF influences thrombin activity by binding to the thrombin cleavage site on both PAR4 and PAR1.

Keywords: Thrombin; PAR4; Bradykinin; Bradykinin 1–5; RPPGF

1. Introduction

Protease-activated receptors (PAR) are novel G-protein coupled receptors that are activated by the proteolysis of an N-terminal exodomain [1]. Upon proteolysis, the newly formed N-terminus acts as a tethered ligand to activate the receptor and initiate multiple signaling cascades via heterotrimeric G-proteins [2,3]. There are four PARs (PAR1–4) that make up this family of proteins. PAR1, 3 and 4 are activated by thrombin, whereas PAR2 is activated by trypsin or tryptase [4]. Thrombin is a major platelet agonist by activating protease activated receptors 1 and 4 (PAR1 and 4) [5]. Thrombin-induced platelet activation initiates signaling pathways that lead to platelet shape change, degranulation, and aggregation. Thrombin activates PAR1 at concentrations ~10-fold less than PAR4, but activation of PAR4 provides a longer stimulus [6,7]. These receptors have distinct roles and both receptors contribute to physiologic hemostasis. A number of PAR1 activation antagonists have been developed [8–10]. However, in order to effectively inhibit human thrombin-induced platelet activation, both PAR1 and PAR4 activation must be blocked.

The terminal angiotensin converting enzyme (ACE) breakdown product of bradykinin, bradykinin 1–5 (BK1–5) or peptide Arg-Pro-Pro-Gly-Phe (RPPGF), inhibits thrombin-induced platelet aggregation, binds to the active site of thrombin and binds to amino acids L[38]DPR[41] in the P4–P1 position on the exodomain of PAR1 to prevent the proteolysis and activation of this receptor [11]. Further, RPPGF and an RPPGF analog, rOicPGF, individually, inhibit the active site of both thrombin and factor VIIa [12]. Additional studies show that RPPGF at 2 mg/kg IP or rOicPGF at 0.44 mg/kg IP significantly delays mouse carotid artery thrombosis in the Rose Bengal Model [12]. Furthermore, both RPPGF and rOicPGF inhibit γ-thrombin induced mouse platelet aggregation in platelet-rich plasma and prolong the bleeding time in the mouse [12]. In contrast to human platelets, mouse platelets do not express PAR1; rather, they express PAR3 and PAR4. In mouse platelets, PAR4 is the signaling receptor, whereas PAR3 is a cofactor for PAR4 activation [13]. Therefore, the ability of RPPGF or rOicPGF to inhibit the activation of mouse platelets suggests that these peptides interact with mouse PAR4 in addition to human PAR1 [11,12]. In this report, we demonstrate that the arginine of the ACE breakdown product of bradykinin, RPPGF, or an analog, rOicPGF, also binds to proline[46] at the P2 position of the exodomain of human PAR4 to inhibit thrombin cleavage of human PAR4 exodomain. These data indicate a single naturally occurring peptide that interferes with thrombin's interaction with both PAR1 and PAR4.

2. Materials and methods

2.1. Materials

The abbreviation used for the synthetic amino acid (2S, 3aS, 7aS)-octahydroindol-2-carboxilic acid is Oic. Standard single capital letter abbreviations are used for L amino acids and lowercase letters are used to designate D amino acids. Peptides RPPGF, scrambled RPPGF, FPRPG and D-Arg-Oic-Pro-Gly-Phe (rOicPGF), Arg-Pro-Pro-Gly-Phe-Lys-biotin (RPPGFK-biotin), biotin-Gly-Arg-Pro-Pro-Gly-Phe (biotin-GRPPGF), RPPGC, SILPAPRGYPGQ (SIL12) and biotin-SIL12 were synthesized by Multiple Peptide Systems, Inc. (San Diego, CA). Peptide Trp-Pro-Pro-Gly-Phe (WPPGF) was synthesized by the Protein and Carbohydrate Structure Core, University of Michigan (Ann Arbor, MI). All peptides used in these studies were greater than >95% pure by reverse-phase HPLC. The purified peptides were

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Abbreviations: RPPGF or BK1–5, bradykinin 1–5; PAR1 & 4, protease-activated receptors 1 & 4; SIL12, peptide SILPAPRGYPGQ

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characterized by analytical HPLC, amino acid analysis, and mass spectrometry for homogeneity. The peptides were colorless, odorless, and soluble in water.

2.2. Cloning and mutagenesis of PAR4 exodomain

The cDNA of human PAR4 from (Gly^{18}-Arg^{78}) was prepared from HEL cell mRNA by RT-PCR and cloned into pET19b or pET31b (Novagen, Madison, WI). The cDNA of human PAR1 exodomain (Ala^{26}-Ser^{99}) has been described [11]. The pET31b constructs were used for bacterial expression of recombinant PAR1 exodomain (PAR1-wt), PAR4 exodomain (PAR4-wt) and PAR4 exodomain mutants because this system produced higher yields of recombinant protein and eliminated the His tag (see below).

Mutations of the PAR4 exodomain were generated by alanine substitution using the PAR4-wt-pET19b as the starting material with the exception of PAR4-P44A/P46A, in which PAR4-P46A was used as the starting material. Mutations were created by using overlapping mutagenizing oligonucleotides and PCR. Two sets of oligonucleotides were used to create each mutant: outside oligonucleotides hybridizing 5′ and 3′ of the coding sequence and internal, overlapping oligonucleotides of opposite orientation containing the mutated nucleotides to change the codons to the desired amino acid residue. The outside primers were T7-promoter (T7p) (5′ end, sense) and T7 terminator (T7t) (3′ end, antisense). The mutagenizing internal oligonucleotides are shown in Table 1.

2.3. Recombinant protein production

Purified recombinant exodomain of PAR1, PAR4, or PAR4 mutants was prepared from 2 liter cultures of *Escherichia coli* BLR(DE3) with the plasmid pET31b containing the PAR4 cDNA that were grown to mid-log phase at 37 °C in Luria broth, followed by induction with 1 mM IPTG for 5 h. After induction, the bacteria were harvested by centrifugation (10 min, $5000 \times g$) and the pellet was resuspended in binding buffer (40 mM Tris–HCl and 500 mM NaCl, pH 7.4) containing 0.3 mg/ml lysozyme. The extracts were then incubated on ice for 15 min for partial lysis and then stored at −80 °C overnight. After thawing to complete the lysis, 1 ml of both RNase A (1 mg/ml) and DNase A (1 mg/ml) was added to the suspension followed by incubation at 37 °C for 1 h. Bacterial lysates were centrifuged at $5000 \times g$ for 15 min and the pellet was resuspended in binding buffer containing 5 mM imidazole and purified with nickel-chelate chromatography followed by treatment with CNBr to remove the KSI fusion protein and the His tag according to the manufacturer's protocol (Novagen, Madison, WI). The final recombinant proteins were further purified by HPLC. The integrity of the expressed PAR4 exodomain was verified by MALDI-TOF mass spectrometry and N-terminal sequencing.

Table 1
Oligonucleotides to prepare PAR4 mutants

PAR4-WT	...S I L **P**^{44}A **P**^{46} R G Y P G Q...[a] 5′-TCAATCCTGCCTGCCCCCCGCGGC-TACCCAGGCCAA-3′[b]
Mutants	Mutagenizing oligonucleotides
S-PAR4-P44A	5′-TCAATCCTG**G**CAGCCCCC-3′
A-PAR4-P44A	3′-GGAC**C**GTCGGGGGGCGCC-5′
S-PAR4-P46A	5′-CCTGCC**G**CACGCGGCTAC-3′
A-PAR4-P46A	3′-TAGGACGGACGG**C**GTGCGCC-5′
S-PAR4-P44A/P46A	5′-CAATCCTG**G**CTGCCGCA-3′
A-PAR4-P44A/P46A	3′-GTTAGGAC**C**GACGGCGT-5′

Overlapping PCR techniques were used to generate PAR4 alanine substitution mutants at Pro^{44}, Pro^{46} or Pro^{44} and Pro^{46} as described in Section 2. Oligonucleotides are labeled "S" for the sense strand and "A" for the antisense strand; the mutated codons are underlined in bold.

[a] Represents the amino acid sequence of the thrombin cleavage site on human PAR4 using the single letter abbreviation code for each amino acid.

[b] Represents the nucleotide sequence of wild type exodomain of human PAR4.

2.4. SDS–PAGE and immunoblotting

PAR exodomains were separated on 16.5 tris–tricine SDS–polyacrylamide gels (BioRad, Hercules, CA) and stained with G250 Coomassie Blue (BioRad) or transferred to nitrocellulose for immunoblotting. Anti-PAR4 peptide polyclonal antibodies were generated in goats by injecting peptide SILPAPRGYPGQ into goats (Quality Control Biochemicals, Hopkinson, MA) [11]. This sequence covers the thrombin cleavage site on the PAR4 exodomain.

2.5. Binding experiments

Peptide RPPGC or PAR4-wt (1 μg/well) in 0.1 M Na_2CO_3, pH 9.6, was bound to microtiter plate wells (F96 CERT.MAXISORP, # 439454, Nunc-Immuno Plate, Fisher Scientific, Chicago, IL) by incubation at 4 °C overnight. Following incubation, wells were washed with 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4, containing 0.05% Tween 20 (PBS-Tween). For experiments in which RPPGC was linked to plates, the wells were blocked with 1% gelatin and the bound RPPGC was incubated with 30 μM biotinylated SIL12 (biotin-SILPAPRGYPGQ) in the presence or absence of various peptides for 1 h at 37 °C. For experiments in which PAR4-wt was linked to plates, the wells were blocked with 1% gelatin and the bound PAR4-wt was incubated with 15 μM RPPGFK-biotin in the presence or absence of various peptides, PAR1-wt, PAR4-wt or PAR4 mutants for 1 h at 37 °C. Following incubation, cells were washed with PBS-Tween and the bound biotin-SIL12 or RPPGFK-biotin was detected by using Immune-Pure streptavidin horseradish peroxidase conjugate (Pierce Chemical Co., Rockville, IL) and peroxidase-specific fast reacting substrate, turbo-3, 3′, 5, 5′-tetramethylbenzidine (turbo-TMB, Pierce) as previously described [14]. The color reaction was stopped by the addition of 1 M phosphoric acid and the reaction was quantitated by measuring the absorbance at 450 nm in a Microplate auto reader EL311 (Bio-Tek, Winooski, VT).

2.6. Proteolysis of PAR4 exodomain

PAR4 exodomain (30 μM) in 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4, was incubated for 15 min at 37 °C with 100–1000 μM rOicPGF [12]. The reaction was initiated by the addition of γ-thrombin (20 nM final concentration) (Haematologic Technologies, Essex Junction, VT). The reaction was stopped at 60 min and separated on SDS–PAGE.

2.7. Hydrolysis of chromogenic substrate

rOicPGF (50 μM) was incubated with γ-thrombin (1 nM) for 5 min at 25 °C. The reaction was initiated by the addition of Sar-Pro-Arg-paranitroanalide (1 mM) (Sigma) and the absorbance was monitored at 405 nm for 16 min.

3. Results

Previous studies showed that the ACE breakdown product of bradykinin, RPPGF, prolonged the bleeding time and delayed the time to thrombosis in a mouse carotid injury model [12]. Since mouse platelets do not express PAR1, these peptides likely inhibited mouse platelet aggregation by blocking mouse PAR4. Investigations were performed to determine if RPPGF and related analogs also bound human PAR4.

A peptide that encompassed the thrombin binding and cleavage site on human PAR4, biotinylated S^{41}ILPAPRGYPGQ^{52} (biotin-SIL12), specifically bound to peptide RPPGC linked to microtiter plates (Fig. 1). The interaction of biotin-SIL12 with RPPGC was blocked by increasing concentrations of soluble RPPGF, rOicPGF, or unlabeled SIL12 with an IC_{50} at ∼1 mM. However, a scrambled peptide of RPPGF, FPRPG, did not interfere with biotin-SIL12 binding to RPPGC (Fig. 1).

Next, recombinant exodomain of PAR4 from Gly^{18}-Arg^{78} was prepared (Fig. 2). On reduced SDS–PAGE, the PAR4

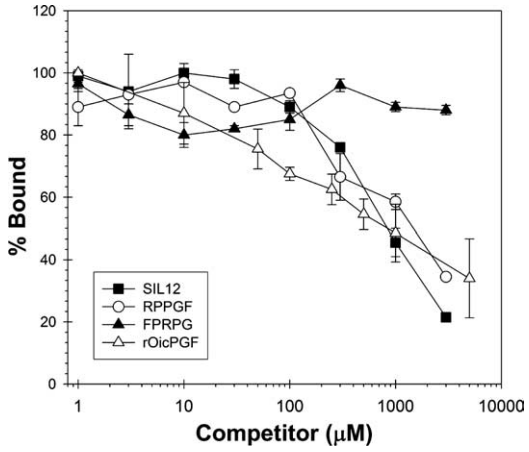

Fig. 1. Inhibition of biotin-SIL12 binding to RPPGC. RPPGC (1 μg/well) in 0.1 M Na_2CO_3, pH 9.6, was linked to microtiter plate wells by overnight incubation at 4 °C. After washing the wells with 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) containing 0.05% Tween and blocking with 1% gelatin, the bound protein was incubated with 30 μM biotin-SIL12 in the absence or presence of increasing concentrations of unlabeled SIL12 (■), RPPGF (○), FPRPG (▲) or rOicPGF (△). The biotin-SIL12 bound to the cuvette well was detected with streptavidin horseradish peroxidase followed by peroxidase specific substrate and quantitated by reading absorbance at 450 nm. The data are normalized to wells containing no inhibitor that represents 100% binding. The data are means ± S.D. of three experiments.

exodomain (PAR4-wt) migrated at ~10 kDa, a position slower than the expected size of 6.44 kDa and there was a minor bands at ~16.9 kDa. To positively identify the major and minor bands seen on SDS–PAGE, N-terminal sequencing and MALDI-TOF mass spectrometry was performed. With the exception of a N-terminal leucine from the expression system, the first nine amino acids of these proteins were identical (LGGTQTPSVY...) and were the expected amino acids from the exodomain of PAR4 starting at position 18, indicating that the upper band is a dimer of the rPAR4 exodomain [15]. On mass spectrometry, there were two significant peaks identified at 6.435 and 12.874 kDa, respectively, corresponding to the expected size of PAR4-wt and its dimer. Additionally, PAR4-wt was recognized by an immunoblot with an anti-PAR4 polyclonal antibody (Fig. 2), but not by antibodies to PAR1 or PAR3 (data not shown).

RPPGFK-biotin was found to specifically bind to exodomain PAR4-wt linked to microtiter plates. This interaction was blocked by soluble RPPGF (IC_{50} = 0.71 mM) or rOicPGF (IC_{50} = 0.45 mM), however, at 1 mM the pentapeptide WPPGF showed 52% less inhibition than the other peptides (Fig. 3). These data indicate that the N-terminal arginine of RPPGF participated in its binding to PAR4-wt. These results were consistent with previous findings that biotin-GRPPGF, that had its N-terminal arginine blocked, did not bind to peptide SIL12 or PAR1-wt exodomain (data not shown). However, the N-terminal arginine was not exclusively involved in binding because at concentrations less than 300 μM there was no difference in the inhibition of binding by WPPGF or the other peptides, indicating that additional amino acids interacted as well.

Since RPPGF binds to PAR4-wt, investigations next mapped the RPPGF binding site on the exodomain of PAR4. Alanine site-directed mutagenesis of the exodomain of PAR4 was performed on Pro^{46} and Pro^{44} adjacent to the thrombin cleavage

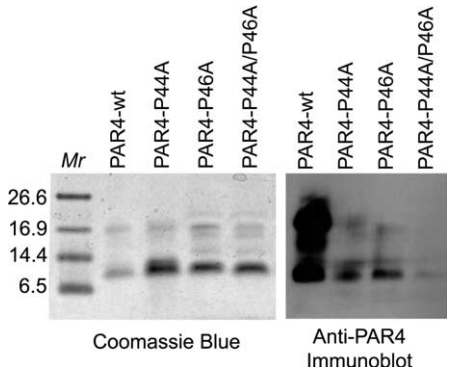

Fig. 2. Expression of wild type and mutant PAR4. Recombinant PAR4-wt and PAR4 mutants were separated by SDS–PAGE and stained with Coomassie G250 (on left) or transferred to nitrocellulose and blotted with anti-PAR4 antibodies (on right). The figure is a representative experiment.

site of PAR4, since RPPGF bound to a peptide that contains the thrombin cleavage site (S^{41}ILPAPRGYPGQ^{52}) (Table 1 and Fig. 1). The recombinant PAR4 alanine substitution mutants were 6410 Da (PAR4-P44A and PAR4-P46A) and 6380 Da (PAR4-P44A/P46A) as determined by mass spectrometry (data not shown) and were recognized by polyclonal antibodies to PAR4 (Fig. 2). However, when both prolines were changed to alanine, there was reduced antigen recognition by the polyclonal antibody reared to peptide SILPAPRGYPGQ, suggesting that the prolines in the peptide were prominent epitopes (Fig. 2). RPPGFK-biotin binding to recombinant PAR4-wt linked to microtiter plates was blocked by soluble PAR1-wt, PAR4-wt, and PAR4-P44A with an IC_{50} of 243, 215, and 235 μM, respectively (Fig. 4). Alternatively, mutagenesis of Pro^{46} either alone or in the presence of the Pro^{44} mutation resulted in forms of recombinant PAR4 that did not compete

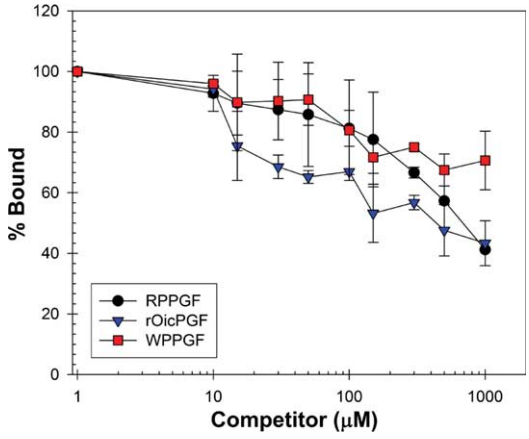

Fig. 3. Inhibition of RPPGFK-biotin binding to PAR4-wt. Recombinant PAR4-wt (1 μg/well) in 0.1 M Na_2CO_3, pH 9.6, was linked to microtiter plate wells by overnight incubation at 4 °C. After washing the wells with 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) containing 0.05% Tween and blocking with 1% gelatin, the bound protein was incubated with 15 μM RPPGFK-biotin in the absence or presence of increasing concentrations (10–1000 μM) of RPPGF (black circle), rOciPGF (blue solid triangle) or WPRPG (red square). The RPPGFK-biotin bound to the cuvette well was detected with streptavidin horseradish peroxidase followed by peroxidase specific substrate and quantitated by reading absorbance at 450 nm. The data are normalized to wells containing no inhibitor that represents 100% binding. The data are means ± S.D. of three experiments.

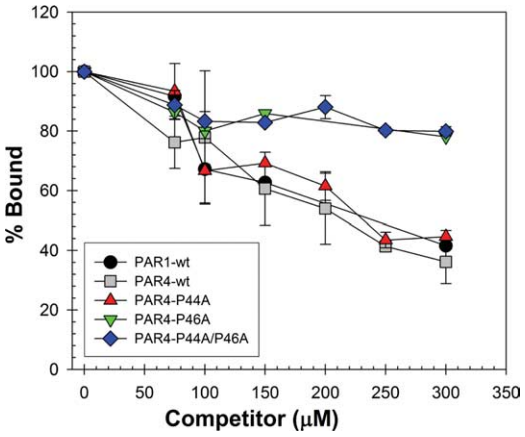

Fig. 4. Inhibition of RPPGFK-biotin binding to PAR4. Recombinant PAR4-wt (1 μg/well) in 0.1 M Na_2CO_3, pH 9.6, was linked to microtiter plate wells by overnight incubation at 4 °C. After washing the wells with 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) containing 0.05% Tween and blocking with 1% gelatin, the bound protein was incubated with 15 μM RPPGFK-biotin in the absence or presence of increasing concentrations of PAR1-wt (black circle), PAR4-wt (grey square), PAR4-P44A (red triangle), PAR4-P46A (green triangle) or PAR4-P44A/P46A (blue diamond). The RPPGFK-biotin bound to the cuvette well was detected with streptavidin horseradish peroxidase followed by peroxidase specific substrate and quantitated by reading absorbance at 450 nm. The data are normalized to wells containing no inhibitor that represents 100% binding. The data are means ± S.D. of three experiments.

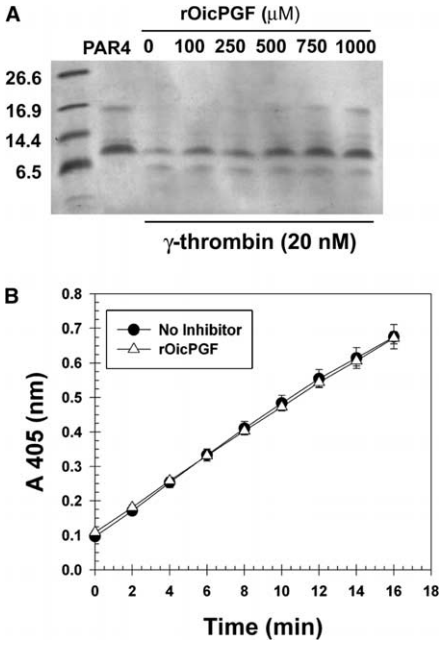

Fig. 5. rOicPGF inhibits proteolysis of the PAR4 exodomain by γ-thrombin. (A) rOicPGF inhibition of γ-thrombin cleavage of PAR4 exodomain. Recombinant PAR4-wt (30 μM) in 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) was incubated with rOicPGF (100–1000 μM) for 15 min at 37 °C. The reactions were initiated by the addition of γ-thrombin (20 nM final concentration) and proceeded for 60 min. The reactions were separated by SDS–PAGE and stained with G250 Coomassie Blue. The lane marked "PAR4" is the undigested starting material. The figure is a representative experiment. (B) No rOicPGF inhibition of γ-thrombin hydrolysis of a chromogenic substrate. γ-thrombin (1 nM) was incubated with (▲) or without (●) rOicPGF (50 μM) for 5 min at 25 °C. The reaction was initiated by the addition of the chromogenic substrate Sar-Pro-Arg-paranitroanalide (1 mM) and hydrolysis of the substrate was monitored over 16 min. These data are means ± standard deviation of three replicates.

RPPGFK-biotin binding to PAR4-wt linked to cuvette wells. These data indicated that Pro^{46} was critical for the interaction of the arginine of RPPGF with the exodomain of PAR4.

Investigations next determined if binding of an analog of RPPGF to the PAR4 exodomain inhibited its proteolysis of PAR4 by thrombin. The exodomain of PAR4 was fully cleaved by 20 nM γ-thrombin in 60 min (Fig. 5A). The proteolysis was progressively inhibited by 100–750 μM rOicPGF (molar ratio of rOicPGF:γ-thrombin is 37 500:1) (Fig. 5A). This effect was completely due to rOicPGF binding to the exodomain of PAR4 because 50 μM rOicPGF did not inhibit 1 nM γ-thrombin hydrolysis of Sar-Pro-Arg-paranitroanalide (molar ratio of rOicPGF:γ-thrombin is 50 000:1) (Fig. 5B). These data indicated that rOicPGF inhibited PAR4 proteolysis by γ-thrombin at concentrations that do not affect the enzymatic activity of γ-thrombin.

4. Discussion

In this report, we demonstrate that the ACE breakdown product of bradykinin, RPPGF, or an analog, rOicPGF, binds to the exodomain of human PAR4 at Pro^{46}, the P2 position, to prevent its proteolysis by thrombin. Further, our studies indicate that the amino terminal arginine of these peptides is essential for this interaction. The interaction of RPPGF with human PAR4 exodomain is consistent with previous studies in which RPPGF and analogs delay the time to thrombosis in mice and inhibit thrombin-induced platelet aggregation in mouse platelets [12].

Bradykinin inhibits thrombin-induced human platelet aggregation [16]. The inhibitory properties of bradykinin map to the peptide RPPGF, a naturally occurring biological peptide that is the terminal ACE breakdown product of bradykinin [16]. Bradykinin, RPPGF and analogs inhibit platelet activation by two mechanisms: they bind to the active site of thrombin and to the exodomain of PAR1 at the thrombin cleavage site [11,16,17]. The present report expands the thrombin inhibitory mechanism of RPPGF and related compounds by showing that they also bind to the exodomain of PAR4. Thus, a naturally occurring peptide binds to thrombin and to PAR1 and PAR4. Recent additional data also indicate that the RPPGF (unpublished) and rOicPGF also inhibit the active site of coagulation factor VIIa if incubated in the absence of tissue factor [12].

The binding site of RPPGF and analogs has been mapped to be near the thrombin cleavage site for both PAR1 and PAR4. There is relatively little sequence identity between PAR1 and PAR4 in this region, so it is unexpected that these peptides bind to both receptors [11,15,18]. These data suggest that there must be similarities in the three-dimensional structure of the PARs in this region that allow these peptides to bind. Using NMR, Cleary et al. [19] have demonstrated that Leu^{43} at P5 of PAR4 ($\underline{L}^{43}$PAPR) is flexible and can exist in two conformations in the presence of thrombin; the side chain of Leu^{43} interacts with Pro^{44} or Pro^{46}. A three-dimensional structure of Leu^{43} interacting with Pro^{46} is similar to the structure of a PAR1 peptide that was co-crystallized with α-thrombin [20].

Specifically, the P5–P2 position of PAR4 (L^{43}PAP) exists in the same conformation as the P4–P1 position of PAR1 (L^{38}DPR) [20]. These data suggest that PAR1 and PAR4 can exist in similar three-dimensional structures, which make it plausible that a single agent can interact with both receptors. In the alanine mutants PAR4-P46A and PAR4-P44A/P46A, it is unlikely that Leu^{43} is able to interact with the alanine at the P2 position and, consequently, the conformation around the P5–P2 position is disrupted resulting in the loss of RPPGF binding. Recognition of the conformational similarity between thrombin substrates is not unique. A monoclonal antibody that inhibits fibrinogen's Aα-chain cross-linking also directly inhibits factor XIIIa [21]. Similarly, just as there is conformational conservation among thrombin substrates, the S1 pockets of serine proteases are also conserved as indicated by the lack of complete substrate specificity of serine proteases on chromogenic substrates. Further, recent evidence suggests that the S1 pockets of thrombin and factor VIIa have similar structures [22]. This information may explain how RPPGF and analogs can interact with PAR1 and PAR4 exodomains as well as the active sites of thrombin and factor VIIa [11,12].

PAR antagonists that have been developed to date prevent the activation of the receptor by interfering with the ligand binding site or specifically interfering with signaling through the intracellular loops [8–10]. In each case, the antagonist acts after the receptor has been proteolyzed by thrombin and thus interferes with the signaling mechanism of the activated receptor. Since the ligand for each of the PARs and the downstream signaling pathways is unique, these kinds of strategies for interfering with the PARs require agents unique for each receptor. In contrast, the mechanism of RPPGF and its analogs' inhibition of PAR activation is quite different. These latter compounds interfere with the proteolysis of both PAR exodomains and, as a result, the activation of both receptors, allowing for a single agent that blocks thrombin-induced platelet activation of both PAR1 and 4.

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