Published in final edited form as:

Biochem Pharmacol. 2008 January 15; 75(2): 438–447.

Characterization of a New Peptide Agonist of the Protease-activated Receptor-1

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Abstract

A new peptide (TFRRRLSRATR), from the c-terminal of human platelet P2Y₁ receptor, was synthesized and its biological function was evaluated. This peptide activated platelets in a concentration-dependent manner, causing shape change, aggregation, secretion and calcium mobilization. Of the several receptor antagonists tested, only BMS200261, a PAR-1 specific antagonist, totally abolished the peptide-induced platelet aggregation, secretion and calcium mobilization. The TFRRR-peptide-pretreated washed platelets failed to aggregate in response to SFLLRN (10 μ M) but not to AYPGKF (500 μ M). In addition, in mouse platelets, peptide concentrations up to 600 μ M failed to cause platelet activation, indicating that the TFRRR-peptide activated platelets through the PAR-1 receptor rather than through the PAR-4 receptor. The shape change induced by 10 μ M peptide was totally abolished by Y-27632, an inhibitor of p160^{ROCK} which is the downstream signal of G_{12/13} pathways. The TFRRR-peptide, YFLLRNP, and the physiological agonist thrombin selectively activated G_{12/13} pathways at low concentrations and began to activate both Gq and G_{12/13} pathways with increased concentrations. Similar to SFLLRN, the TFRRR-peptide caused phosphorylation of Akt and Erk in a P2Y₁₂ receptor-dependent manner, and p-38 MAP kinase activation in a P2Y₁₂-independent manner. The effects of this peptide are elicited by the first six amino acids (TFRRRL) whereas the remaining peptide (LSRATR), TFERRN, or TFEERN had no effects on platelets. We conclude that TFRRRL activates human platelets through the PAR-1 receptors.

Keywords

PAR agonists; P2Y₁ receptor; Gq; G_{12/13}; MAP kinases; calcium mobilization

1. INTRODUCTION

Platelets are a fundamental component of the hemostatic process. Platelet activation leads to shape change, aggregation, secretion from granules, and finally in the formation of a stable clot at the damaged endothelium surface [1]. Physiologically platelet activation occurs primarily by collagen in the subendothelium, but is rapidly amplified by recruitment of platelets to the site of the injury [2-4]. Adenosine diphosphate (ADP), released from the dense granules,

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thromboxane A₂, produced in the activated platelets, and thrombin, generated on the activated platelet surface, are key players in the amplification response [5-7].

Thrombin activates platelets through cell surface receptors known as protease-activated receptors (PARs) [8]. PARs belong to the class of seven transmembrane domain G protein-coupled receptors. Among the four members of PARs characterized to date, PAR-1, PAR-3 and PAR-4 are activated by thrombin and considered primarily as thrombin receptors [9]. Thrombin activates the PARs through an unique mechanism involving proteolytic cleavage of the amino terminal of the receptor and thus exposing a new amino terminus. The newly formed amino terminus binds to the extracellular domain of the receptor to cause intracellular signaling [10,11]. Numerous studies on the structure-function analysis of PAR1 agonists have revealed a requirement of free amine group at the amino terminus, a short chain amino acid at position 1, aromatic amino acid at position 2 and arginine at position 5 for potent activation of PAR-1 [12,13].

Human platelets express PAR-1 and PAR-4, whereas the mouse platelets express PAR-3 and PAR-4 [10,14,15]. In human platelets, PAR-1 is a high-affinity receptor that is activated at low concentrations of thrombin, and PAR-4 is a low-affinity receptor that mediates thrombin signaling at higher concentrations [14]. In mouse platelets, PAR-3 serves as a co-receptor for PAR-4, wherein thrombin binds to PAR-3 and PAR-4, and cleaves the amino terminus of PAR-4 [16].

In order to evaluate the physiological and pathophysiological functions of the protease-activated receptors, specific peptide agonists, which mimic the actions of the newly exposed N-terminal, were synthesized. These activation peptides are based on the tethered ligand of the PARs, and hence are specific for the PARs. For example, SFLLRN, which mimics the new N-terminal amino terminal activates human PAR-1 and induces human platelet aggregation and degranulation without cleavage of PAR-1 receptor [10,17]. Resmussen et al find the peptide YFLLRNP works as a partial PAR-1 agonist that induces platelet shape change without calcium mobilization; however, full activation of platelets is achieved only at higher concentrations [18]. The shape change caused by 60 μ M YFLLRNP appears to be mediated by the G_{12/13} pathway through activation of RhoA and p160^{ROCK}. Consistently, Y-27632, a p160^{ROCK} inhibitor, is reported to totally block the stimulatory effects of YFLLRNP [19,20].

In the current study, we evaluated the biological function of a synthetic peptide TFRRRLSRATR, which is derived from the carboxy terminus of P2Y₁ receptor that is important for Gq-coupling. Instead of interfering with the P2Y₁ receptor, this peptide is able to directly activate human platelet through PAR-1 receptor. Unlike the known PAR-1 peptide agonists, this peptide sequence is not derived from the amino terminus region of the receptor. Its structural diversity from the other peptide agonists of PAR-1 and its dose-dependent signaling effects make this peptide a distinct agonist that will help us in understanding the molecular mechanisms of ligand-receptor interactions.

2. METHODS

2.1 Materials

Peptide (TFRRRLSRATR) was synthesized by New England Peptide, Inc. (Gardner, MA). Luciferin-luciferase reagent was purchased from Chrono-Log (Havertown, PA). Hexapeptides SFLLRN and AYPGKF were custom synthesized at Invitrogen (Carlsbad, CA). AR-C69931MX was a gift from Astra-Zeneca Research Laboratories (Charnwood, Loughborough, United Kingdom). The PAR-1 antagonist BMS-200261 was obtained as a generous gift from Dr. Steven Seiler (Bristol-Myers Squibb). Fura-2 AM was from Invitrogen (Eugene, OR). The p160^{ROCK} inhibitor Y-27632 and Prostaglandin E₁ (PGE₁) were from Biomol Research

Laboratories (Plymouth Meeting, PA). Anti-phospho-Akt (Ser473), anti-phospho-Erk and anti-phospho-p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA). Alkaline phosphatase-labeled secondary antibody was from Kirkegaard & Perry Laboratories (Gaithersburg, MD). CDP-Star chemiluminescent substrates were purchased from Applied Biosystems (Foster City, CA). PI-3 kinase inhibitors wortmannin and LY294002 were from Biomol Research Laboratories (Plymouth Meeting, PA). MRS-2179, apyrase grade VII, thrombin and acetylsalicylic acid were obtained from Sigma (St. Louis, MO). All the other reagents were of reagent grade, and de-ionized water was used throughout.

2.2 Washed human platelet preparation

Washed human platelets were prepared as described previously, under the guidelines approved by the Institutional Review Board [21]. Whole blood from healthy, drug-free donors at the Sol Sherry Thrombosis Research Center of Temple University (Philadelphia, PA) was collected with informed consent, in tubes containing acid-citrate-dextrose (ACD: 2.5 g sodium citrate, 1.5 g citric acid, and 2 g glucose in 100 mL deionized water). Citrated blood was centrifuged to obtain platelet-rich plasma (PRP). The PRP was treated with 1 mM acetylsalicylic acid (aspirin) to block thromboxane A₂ production [22] and centrifuged to get the platelet pellet. Platelet pellet was resuspended in calcium-free Tyrode's buffer.

2.3 Preparation of washed mouse platelets

Blood was collected from anesthetized mice, in accordance with the IACUC approved protocols, by cardiac puncture into syringes containing 3.8% sodium citrate as anticoagulant. The whole blood was centrifuged (IEC Micromax centrifuge; International Equipment, Needham Heights, MA) at 100 x relative centrifugal forces (RCF) for 10 minutes to isolate the PRP. Prostaglandin E₁ (PGE₁, 1 mM) was added to PRP. The PRP were centrifuged at 400 RCF for 10 minutes and the pellet was resuspended in Tyrode buffer containing 0.01 unit/mL apyrase.

2.4 Platelet aggregation and secretion

Aggregation and secretion of 0.5 mL washed platelets was analyzed using a P.I.C.A. lumiaggregometer (Chrono-log, Havertown, PA). Aggregation was measured by light transmission under stirring conditions (900 rpm) at 37°C. ATP secretion from platelet-dense granules was measured by using the Luciferin-Luciferase assay and the platelets were stimulated in a lumiaggregometer at 37°C with stirring at 900 rpm and the corresponding luminescence was measured. Agonists were added simultaneously for platelet stimulation and platelet were preincubated with each inhibitor at 37°C. The aggregation and secretion data are represented in the form of actual tracings [20].

2.5 Platelet desensitization

Platelet desensitization was performed as described earlier [23]. Briefly, washed human platelets were incubated for 40 minutes at 37°C without stirring in the absence or presence of 300 µM peptide. Immediately after incubation, platelets were assayed for their responses to various agonists.

2.6 Intracellular Calcium Measurements

For intracellular calcium mobilization studies, PRP was incubated with 2 µM fura-2 with acetylsalicylic acid (aspirin) for 45 minutes and leave in room temperature for another 15 minutes. Platelets were then isolated and washed as described above. Changes in fluorescence were measured using Aminco-Bowman Series 2 luminescence spectrometer with a water-jacketed cuvette holder, equipped with a thermostat, at 37°C and set at constant stirring. 0.5 mL sample were analyzed with an excitation wavelength of 340 nm and an emission

wavelength of 510 nm. Fluorescence measurements were converted to calcium concentrations using the equation reported by Grynkiewicz et al. [24], where $F_{\min}$ and $F_{\max}$ were determined with each respective platelet preparation [25].

2.7 Western blotting

Washed human platelets (0.2 mL) were stimulated with agonists under non-stirring conditions for 3 minutes and lysed using $3 \times$ sample loading buffer and boiled for 5 minutes. The platelet lysates were loaded on to a 10% Tris-glycine gel, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane. Nonspecific binding sites were blocked by incubation in blocking buffer (5% nonfat dry milk, 3% bovine serum albumin (BSA), 20 mM Tris, 140 mM NaCl) for 1 hour at room temperature (RT) under rocking conditions and then the membrane was incubated with primary antibody (1:1000 dilution for phospho-Akt, phospho-Erk and phospho-p38MAP kinase in TBST, 2% BSA) overnight with gentle rocking. After three 5-minute washes with TBST, the membrane was probed with alkaline phosphatase-labeled goat anti-rabbit IgG (1:5000 dilution in TBST with 2% BSA) for 1 hour at RT. The membrane was washed 3 times for 5 minutes each time using the $1 \times$ TBST, once with deionized water and last with $1 \times$ tropix buffer (Tropix, Bedford, MA). Membrane was then incubated with CDP-Star chemiluminescent substrate for 15 minutes at RT and immunoreactivity was detected using Fujifilm Luminescent Image Analyzer (Fujifilm Medical Systems, Stamford, CT).

3. RESULTS

Recently we have identified a domain, TFRRRLSRATR, in the carboxy terminus of the P2Y₁ receptor that is important for Gq coupling [26]. In efforts to develop intracellular antagonists to P2Y₁ receptor, we tested the ability of this peptide to competitively interfere with P2Y₁ receptor's ability to modulate Gq pathways using platelets as an experimental system. To facilitate the transport of the peptide across plasma membrane, an amino terminal palmitoylation was introduced. To our surprise, this palmitoylated peptide activated human platelets. Hence we began characterizing this peptide to understand the underlying mechanisms of platelet activation by this novel peptide.

3.1 TFRRR-peptide induced responses in human platelets

We first evaluated whether the peptide without the lipid modification at the N-terminus would activate platelets. The non-palmitoylated TFRRR-peptide activated platelets in a concentration-dependent manner. At low concentrations (10 μ M) this peptide induced platelet shape change, and with increasing concentrations (100- 400 μ M), this peptide caused full platelet aggregation (Figure 1A). However, control peptide with scrambled sequence (RTARSLRRRFT) failed to cause either shape change or aggregation even at 1 mM (Fig. 1A). The TFRRR-peptide, however, did not cause any mobilization of calcium from intracellular stores at 10 μ M. This peptide caused calcium mobilization in human platelets starting at 30 μ M (Fig. 1B). The maximum increases in intracellular calcium occurred at 300 μ M, the concentration at which maximum platelet aggregation also occurred.

3.2 Evaluation of the role of PARs in the TFRRR-peptide-induced platelet activation

In order to investigate the mechanism of TFRRR peptide-induced platelet activation, we tested the effect of different platelet receptor specific antagonists on TFRRR-peptide-induced platelet aggregation. As this peptide caused mobilization of calcium from the intracellular stores, we evaluated the role of Gq coupled receptors in platelet aggregation induced by this peptide. Because peptide agonists activate PARs on platelets, we hypothesized that this TFRRR-peptide elicits its responses through PARs on platelets. Consistent with our hypothesis, SQ29548, a thromboxane receptor antagonist, or MRS2179, a P2Y₁ receptor antagonist did not have any

effect on the TFRRR-peptide-induced platelet aggregation (Fig. 2A). Of the PAR specific antagonists, BMS200261, a PAR-1 specific antagonist, completely abolished the TFRRR-peptide-induced platelet aggregation, secretion and calcium mobilization (Fig. 2B). These data indicated that this peptide might activate platelets through the PAR-1 receptors. To further test our hypothesis, we desensitized the platelets by pre-treatment of washed human platelets with 300 μ M TFRRR-peptide for 40 minutes and then stimulated these platelets with either SFLLRN (a PAR-1 selective agonist) or AYPGKF (a PAR-4 selective agonist). The human platelets, pretreated with the peptide, failed to aggregate in response to SFLLRN (10 μ M) but aggregated normally in response to AYPGKF (500 μ M) (Fig. 3A).

To further confirm these results, we used washed mouse platelets, which are devoid of the PAR-1 receptor. In mouse platelets, TFRRR-peptide failed to cause platelet aggregation, up to a concentration of 600 μ M (Fig. 3B). These results imply that TFRRR-peptide activates human platelets by activating PAR1. Since mouse platelets lack PAR1, this peptide fails to activate mouse platelets.

3.3 Differential activation of Gq and G12/13 pathways by TFRRR-peptide and thrombin

As the previous results showed that TFRRR-peptide lacking lipid modification activates PAR1 on the cell surface, we used the TFRRR-peptide without the N-terminal palmitoylation in the subsequent studies. Offermanns et al have shown that thrombin and thromboxane activate Gq and G_{12/13} pathways [27]. In the Gq null mouse platelets, thrombin and thromboxane cause shape change through RhoA-p160^{ROCK} pathway. As we have observed that TFRRR-peptide causes platelet shape change at 10 μ M concentration without causing increases in intracellular calcium levels, we evaluated the role the RhoA-p160^{ROCK} pathway in this calcium-independent shape change. As shown in Fig. 4, 10 μ M TFRRR-peptide-induced platelet shape change was abolished by a p160^{ROCK} inhibitor, Y27632. These data indicate that at 10 μ M concentrations the TFRRR-peptide selectively activates G_{12/13} pathways, leading to the activation of RhoA-p160^{ROCK} pathways. The results indicate that the TFRRR-peptide activates the G12/13 and Gq pathways selectively in a concentration-dependent manner. At low concentrations, the TFRRR-peptide activated only G_{12/13} pathways in platelets, but with the increasing concentrations, this peptide also activates Gq pathways and causes full platelet activation.

We evaluated whether differential activation of G12/13 and Gq pathways occurs when platelets are activated by physiological agonist thrombin. As shown in Fig. 4, very low concentrations (0.005 U/ml) of thrombin, similar to YFLLRNP or TFRRR-peptide, cause platelet shape change that is totally blocked by p160^{ROCK} inhibitor Y27632, indicating that such low concentrations of thrombin selectively activate only G12/13 pathways. As the concentration of thrombin increased to 0.01 and 0.02 U/ml, thrombin-induced platelet shape change is inhibited but not abolished by Y27632 (Fig. 4), indicating that Gq pathways are also activated by these concentrations of thrombin. Thus, thrombin also activates G12/13 and Gq pathways differentially based on the concentration.

3.4 Role of secreted ADP in the PAR-1 activating peptide-induced platelet irreversible aggregation

Trumel et al have shown that secreted ADP acting through the P2Y₁₂ receptor contributes to the irreversible aggregation by SFLLRN [28]. We evaluated the role P2Y₁₂ receptor in TFRRR-induced platelet irreversible aggregation, using AR-C69931MX, a P2Y₁₂ receptor selective antagonist. In the absence of AR-C69931MX, TFRRR-peptide as well as SFLLRN caused irreversible aggregation. However, in the presence of AR-C69931MX, the aggregation induced by both SFLLRN and TFRRR-peptide became reversible (Fig. 5A). As PI-3 kinases are important for promoting irreversible aggregation, we evaluated whether they also have a

role in TFRRR-peptide-induced irreversible aggregation. As shown in Fig. 5B, wortmannin and LY294002 converted irreversible aggregation induced by TFRRR-peptide and SFLLRN to reversible aggregation. These results indicate that the TFRRR-peptide activates PAR-1 in a similar manner as SFLLRN.

3.5 Activation of Akt, Erk2 and p-38 MAPK by TFRRR-peptide in human platelets

We have shown that the activation of P2Y₁₂ receptor and Gi pathways by secreted ADP is required for PAR-mediated Akt phosphorylation in platelets [29,30]. Furthermore, we have shown that PAR-mediated Erk phosphorylation is potentiated by secreted ADP through the P2Y₁₂ receptor [31]. Hence we evaluated the role of P2Y₁₂ receptor in TFRRR-peptide-induced phosphorylation of Akt and Erk. As shown in Fig. 6, TFRRR-peptide or SFLLRN-induced Akt phosphorylation (Fig. 6A), and Erk phosphorylation (Fig. 6B) are inhibited by AR-C69931MX. However, p38 MAP kinase activation by SFLLRN or TFRRR-peptide was unaffected by AR-C69931MX, indicating that PAR-mediated p38 MAP kinase activation occurs independently of the P2Y₁₂ receptor (Figure 6C). These data indicate that TFRRR-peptide-induced platelet activation is due to PAR-1 activation.

3.6 Structure-function studies on the TFRRR-peptide

In order to evaluate the importance of specific amino acids in TFRRR-peptide, we also synthesized a series of peptides. Figure 7 shows that scrambled sequence RTARSLRRRFT failed to cause shape change or aggregation at 300 μ M, a concentration at which the TFRRR peptide elicits a range of responses. In addition, the first part of TFRRR-peptide (TFRRRL) has the similar effect as TFRRR-peptide, whereas the second part of TFRRR-peptide (LSRATR) failed to cause platelet aggregation and calcium mobilization (Fig. 7), which indicates that the first part of TFRRR-peptide is the key for the platelet activation. Interestingly, peptide TFRRRN, with a change in the last amino acid from Leu to Asn, also is able to activate the platelets. If the third or fourth Arg is altered to Glu, the peptide failed to elicit any platelet activation (Fig. 7). All of these data indicate that the effect of TFRRR-peptide depended on the first six amino acids and the third and fourth amino acids, rather than the sixth amino acid, are essential for this peptide-induced platelet activation.

4. DISCUSSION

Platelet activation plays a key role in the pathophysiology of thrombotic diseases [1,32-34]. The major receptors on the platelet membrane include protease-activated receptors (PARs) and ADP receptors [35,36]. In platelets there are two ADP receptors: Gq-coupled P2Y₁ and Gi-coupled P2Y₁₂ receptors [37,38]. Coactivation of both receptors is required for ADP-mediated full activation of platelets as determined by shape change and aggregation [37]. ADP receptors belong to the seven-transmembrane domain G-protein-coupled receptor (GPCR) family. We have recently identified that a ten amino acid domain in the carboxy terminus of the P2Y₁ receptor that is important for Gq stimulation [26]. The cell-penetrating peptides containing i3 loop peptides derived from protease-activated receptors PAR-1 and PAR-4 inhibit thrombin-mediated platelet aggregation [39,40]. Based on these studies we investigated whether we could block P2Y₁-Gq coupling using a cell penetrating peptide from this domain (TFRRRLSRATR). To our surprise, we saw that this peptide activated platelets. Although we initially thought that the peptide might be directly stimulating Gq pathways upon entering the cell, we ruled out the possibility by synthesizing the same peptide without the palmitoylation. The peptide thus made was also able to activate platelets suggesting that the peptide activates one of the cell surface receptors. Hence we began characterizing the mechanism of platelet activation by this peptide (TFRRRLSRATR).

The TFRRR- peptide caused concentration-dependent platelets shape change, aggregation, secretion and calcium mobilization. In order to investigate the mechanism of this peptide, we used different platelet receptor specific antagonists and found that peptide-induced platelet activation was totally abolished by PAR-1 specific antagonist BMS200261. If we desensitize the platelet receptors by using 300 μ M of the peptide, platelets fail to respond to SFLLRN but not to AYPGKF. Furthermore, in mouse platelets, which are devoid of the PAR-1 receptor, peptide concentrations up to 600 μ M failed to cause platelet activation. In addition, blockade of PI-3 kinase rendered platelet aggregation induced by TFRRR-peptide reversible. These effects are similar to those observed with SFLLRN under similar conditions. Finally, the peptide caused activation of Akt, Erk, and p38 kinases in a similar manner as PAR-1 activating peptide, SFLLRN. These results clearly demonstrate that this new peptide specifically activates platelets through the PAR-1 receptor, and does not stimulate PAR-4 receptor.

Notwithstanding the similarities between SFLLRN and TFRRR peptide functions, the latter peptide at low concentration acts as a partial agonist similar to YFLLRN and activates G12/13 pathways [29]. At higher concentrations, however, it mimics SFLLRN by activating G12/13 and Gq pathways together. In this respect, the TFRRR-peptide resembles the YFLLRNP peptide, which also activates G12/13 and Gq pathways in a concentration-dependent manner (although TFRRR peptide is relatively more potent of the two). Thus, the TFRRR-peptide, like other PAR agonists (including thrombin), traffics signal through G12/13 and Gq pathways in a concentration-dependent fashion. However, physiologically these peptides do not exist and the PARs are activated by proteases. Upon proteolytic activation by thrombin, the tethered ligand intramolecularly activates the receptor [10,11]. Thus, the concentration of the tethered ligand should be the same physiologically. A possible explanation for such a selective activation of G12/13 signaling pathways may be that PARs coupled to G12/13 proteins exist in a favorable conformation for binding and subsequent cleavage by thrombin relative to the Gq associated PARs. Thus at low concentrations of thrombin these G12/13-coupled PARs are stimulated and transduce signal before Gq-coupled PARs become activated.

Another interesting observation is that the palmitoylated TFRRR-peptide activated platelets (data not shown). Previous studies have indicated that capping the amino terminus with acetyl moiety of peptide agonists completely abolished PAR1 activation [13]. Thus it is not clear at this point whether the N-terminal lipid modified TFRRR-peptide and the non lipid modified TFRRR-peptide activate platelets through identical mechanisms.

PAR-1 is a high affinity thrombin receptor in human platelets and it is activated by serine protease, such as thrombin [14]. After activation, it will cleave its extracellular N-terminus and expose a new N-terminal (S^{42} FLLRNPNDK 51) acting as a tethered ligand to bind the receptor surface C-terminal residues P 85 AFIS 89 , which is named as PAR-1 binding site-1 (LBS-1) [41]. The intramolecular interaction of ligand residue Phe 43 and LBS-1 residue Ser 89 , and the hydrophobic contact of LBS-1 residue Ile 88 and ligand residue Leu 44 both are crucial for the ligand binding and receptor activation and mutation any of these residues will cause dramatically decrease of the receptor activation induced by peptide agonist, whereas the N-terminal of the ligand Ser 42 is not required for agonist activity and does not contact with binding site-1 [41,42]. Thus, the two PAR1 activating peptides SFLLRN and YFLLRNP are very similar in their sequence and have the crucial Phe and Leu residues. The difference between YFLLRNP and SFLLRN is that they have the different N-terminal residue, which suggests that a specific N-terminal is not necessary for agonist activation. The amino terminal may influence the agonist activation by binding to other residues of extracellular domains of the receptors. Thus, YFLLRNP is a partial agonist at the PAR-1 whereas SFLLRN is a full agonist.

The peptides used in our study have only Phe residue that presumably interacts with the Ser 89 residue in the LBS-1 of PAR-1. The Leu at fourth position was proposed to be important

for activation [46] with a preference for larger hydrophobic residues [48]. The TFRRR-peptide activates PAR-1, although it lacks the Leu residue that interacts with the Ile⁸⁸ in the PAR-1. It is interesting that changing of either of the two basic Arg residues to an acidic residue made the peptide inactive. Thus it appears that the Leu⁴⁴ could be substituted with Arg residue and hence is not essential for the activity of the peptide. It is interesting that the corresponding residue to Leu⁴⁴ in murine and hamster PAR1 is Phe [10,11,43-45]. In addition, mutation of this residue to a proline did not affect the activity of the peptide [46]. However, chemical modification of this residue (N-Me-L) or introduction of rigid spacers in this position resulted in the loss of activity [46]. Thus it appears that there is some flexibility for residues in the third position.

The consensus sequence among all the human PAR-1 activating peptides, including those used this study, is XFXXR, indicating the importance of the second residue (Phe) and fifth residue (Arg). Earlier work from Brass and co-workers has identified these two residues as the most important in the PAR-1 activating peptides [43]. Among various species, these two residues (Phe and Arg) are highly conserved in the PAR-1 [10,11,44,45,47]. However, in the model proposed by Seeley et al, the R⁴⁶ (fifth residue in the peptide) does not interact with the LBS-1 [41]. These issue needs to be resolved in the future.

Thrombin receptors are the important molecular targets for anti-thrombotic drugs. In human platelets, PAR-1 is the predominant thrombin receptor [14]. Several peptide or non-peptide agonists and antagonist based on its unique activating mechanism were synthesized to evaluate the function of PAR-1. The peptide we synthesized further confirmed that two residues (Phe and Arg) are critical for the agonist binding and activation. This work will enable us to find the smallest molecular PAR-1 ligand to initiate the PAR-1 activation.. In summary, we report novel structural requirements for PAR 1 agonists and a signal trafficking downstream of PAR1 by a single agonist.

Acknowledgements

We thank Dr. G. L. Prasad, Temple University School of Medicine, for critically reviewing the manuscript. This work is supported by HL81322, HL80444 and HL60683 from National Institutes of Health (SPK).

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Figure 1. TFRRR-peptide causes platelet aggregation and calcium mobilization

Washed aspirin-treated human platelets were stimulated with unmodified TFRRR-peptide (3-400 μ M) or 1 mM scrambled peptide (RTARSLRRRFT) under stirring conditions at 37°C. In panel A, platelet aggregation was determined by measuring light transmittance. Tracings were representative of at least three separate experiments. In panel B, Calcium levels were analyzed. Each bar was the average of three experiments $\pm$ SEM from three different donors.

Figure 2. The effect of receptor agonists on TFRRR-peptide induced platelet activation
 Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (300 μ M), in the presence and absence of SQ29548 (5 μ M), MRS2179 (100 μ M) and BMS200261 (20 μ M) (panel A) under stirring conditions at 37°C. Panel B, Calcium mobilization was tested in human

platelets stimulated with TFRRR-peptide (300 μ M) and with or without BMS200261 (20 μ M). Tracings were representative of at least three separate experiments.

A

Figure 3. The role of PAR-1 in the TFRRR-peptide-induced platelet activation

Washed human platelets were incubated at 37 °C for 40 min in the absence or presence of 300 μ M peptide (panel A), followed by stimulation with TFRRR-peptide (300 μ M), SFLLRN (10 μ M) or AYPGKF (500 μ M) to initiate aggregation. Tracings were representative of at least three separate experiments. In panel B, Effect of TFRRR-peptide on mouse platelet was tested. Washed mouse platelets were stimulated with AYPGKF (500 μ M) and TFRRR-peptide (300 and 600 μ M) under stirring conditions at 37°C. Platelet aggregation was determined by measuring light transmittance. ATP secretion from platelet dense granules was measured by using the Luciferin-Luciferase assay. Tracings were representative of at least three experiments.

Figure 4. Low concentration of TFRRR-peptide or thrombin causes G12/13 pathway activation
Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (10 μ M), YFLLRNP (60 μ M), or different concentrations of thrombin in the presence and absence of Y26732 (10 μ M) under stirring conditions at 37°C. Tracings were representative of at least three separate experiments.

Figure 5. Role of secreted ADP and PI3-K in the PAR-1 activating peptide induced platelet irreversible aggregation

Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (300 μ M) and SFLLRN (10 μ M), in the presence or absence of AR-C69931MX (100 nM) (panel A), wortmannin (100 nM) and LY294002 (25 μ M) (panel B) under stirring conditions at 37°C. Tracings were representative of at least three separate experiments.

Figure 6. Role of P2Y₁₂ receptor in TFRRR-peptide induced phosphorylation of Akt, Erk and p38MAPK

Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (300 μ M) or SFLLRN (10 μ M) for 3 minutes, in the presence or absence of P2Y₁₂ antagonist AR-C69931MX (100 nM). Akt phosphorylation (Panel A), Erk Phosphorylation (Panel B) and

p-38 MAPK (Panel C) phosphorylation were measured by western blot analysis. The data are representative of experiments done using platelets from at least three different donors.

Figure 7. Structure-function studies on TFRRR-peptide

Washed aspirin-treated human platelets were stimulated with different peptides derived from the TFRRR-peptide under stirring conditions at 37°C. In panel A, platelet aggregation and in panel B calcium release were analyzed. (TFRRR-peptide: TFRRRLSRATR; scrambled peptide: RTARSLRRRFT; first part of TFRRR-peptide: TFRRRL; second part of TFRRR-peptide: LSRATR; point mutant peptide: TFRRRN, TFERRN, TFEERN). Tracings were representative of at least three separate experiments.