BIOCHEMICAL PHARMACOLOGY 75 (2008) 438–447

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Characterization of a new peptide agonist of the protease-activated receptor-1

Yingying Mao [a], *Jianguo Jin* [a], *Satya P. Kunapuli* [a,b,c,*]

[a] *Department of Physiology, Temple University School of Medicine, Philadelphia, PA, USA*
[b] *Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA, USA*
[c] *Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA, USA*

ARTICLE INFO

Article history:
Received 11 July 2007
Accepted 4 September 2007

Keywords:
PAR agonists
$P2Y_1$ receptor
Gq
G12/13
MAP kinases
Calcium mobilization

ABSTRACT

A new peptide (TFRRRLSRATR), derived from the c-terminal of human platelet $P2Y_1$ 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 protease activated receptor 1 (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 μM) but not to AYPGKF (500 μM). In addition, in mouse platelets, peptide concentrations up to 600 μM 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 μM peptide was totally abolished by Y-27632, an inhibitor of $p160^{ROCK}$ which is a downstream mediator of G12/13 pathways. The TFRRR-peptide, YFLLRNP, and the physiological agonist thrombin selectively activated G12/13 pathways at low concentrations and began to activate both Gq and G12/13 pathways with increasing concentrations. Similar to SFLLRN, the TFRRR-peptide caused phosphorylation of Akt and Erk in a $P2Y_{12}$ receptor-dependent manner, and p-38 MAP kinase activation in a $P2Y_{12}$-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 PAR-1 receptors.

Published by Elsevier Inc.

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, 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

* *Corresponding author at*: Department of Physiology, Temple University, Rm. 224OMS, 3420 N. Broad Street, Philadelphia, PA 19140, USA. Tel.: +1 215 707 4615; fax: +1 215 707 4003.
E-mail address: spk@temple.edu (S.P. Kunapuli).
0006-2952/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.bcp.2007.09.002

by thrombin and considered primarily as thrombin receptors [9]. Thrombin activates the PARs through a 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 a 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 activates human PAR-1 and induces human platelet aggregation and degranulation without cleavage of PAR-1 receptor [10,17]. Rasmussen 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 μM YFLLRNP appears to be mediated by the G12/13 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 $P2Y_1$ receptor that is important for Gq-coupling. Instead of interfering with the $P2Y_1$ 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 E_1 (PGE_1) 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 and 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× relative centrifugal forces (RCF) for 10 min to isolate the PRP. Prostaglandin E_1 (PGE_1, 1 mM) was added to PRP. The PRP were centrifuged at 400 RCF for 10 min 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 were 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 platelets were preincubated with each inhibitor at 37 °C. The aggrega-

tion 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 min 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 min and left in room temperature for another 15 min. 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 was 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 min and lysed using 3× sample loading buffer and boiled for 5 min. The platelet lysates were loaded on to a 10% Tris-glycine gel, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane. Non-specific 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 h 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-min 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 h at RT. The membrane was washed 3 times for 5 min each time using the 1× TBST, once with deionized water and last with 1× tropix buffer (Tropix, Bedford, MA). Membrane was then incubated with CDP-Star chemiluminescent substrate for 15 min 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 $P2Y_1$ receptor that is important for Gq coupling [26]. In efforts to develop intracellular antagonists to $P2Y_1$ receptor, we tested the ability of this peptide to competitively interfere with $P2Y_1$ 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 μM) this peptide induced platelet shape change, and with increasing concentrations (100–400 μM), this peptide caused full platelet aggregation (Fig. 1A). However, a 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 μM. This peptide caused calcium mobilization in human platelets starting at 30 μM (Fig. 1B). The maximum increases in intracellular calcium occurred at 300 μM, 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 $P2Y_1$ 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 μM TFRRR-peptide for 40 min 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 μM) but aggregated normally in response to AYPGKF (500 μM) (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 μM (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.

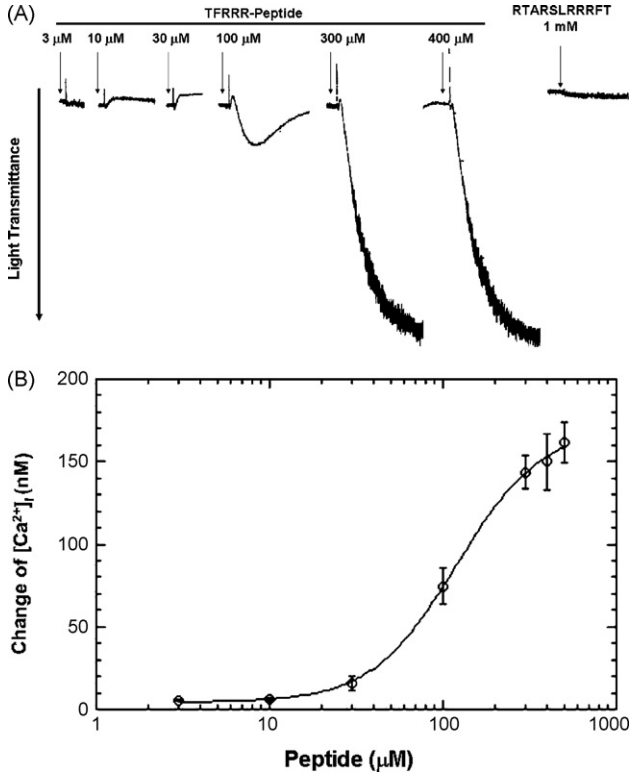

Fig. 1 – TFRRR-peptide causes platelet aggregation and calcium mobilization. Washed aspirin-treated human platelets were stimulated with unmodified TFRRR-peptide (3–400 μM) or 1 mM scrambled peptide (RTARSLRRRFT) under stirring conditions at 37 °C. In panel A, platelet aggregation was determined by measuring 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 ± S.E.M. from three different donors.

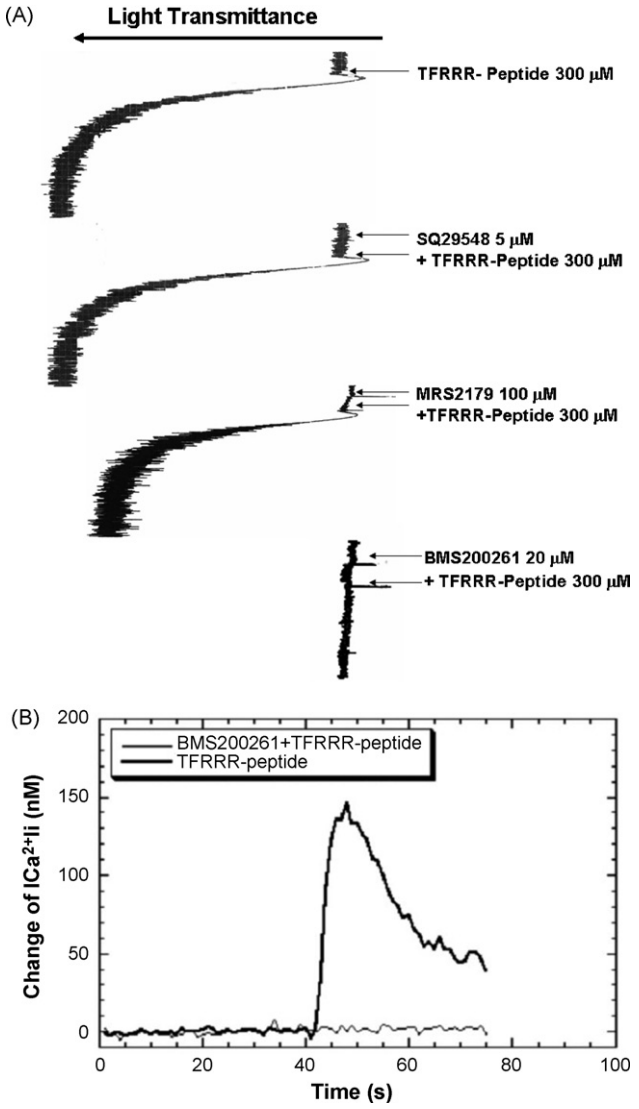

Fig. 2 – The effect of receptor agonists on TFRRR-peptide induced platelet activation. Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (300 μM) in the presence and absence of SQ29548 (5 μM), MRS2179 (100 μM) and BMS200261 (20 μM) (panel A) under stirring conditions at 37 °C. (Panel B) Calcium mobilization was tested in human platelets stimulated with TFRRR-peptide (300 μM) and with or without BMS200261 (20 μM). Tracings were representative of at least three separate experiments.

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 G12/13 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 μM concentration without causing increases in intracellular calcium levels, we evaluated the role of RhoA-p160^{ROCK} pathway in this calcium-independent shape change. As shown in Fig. 4, 10 μM TFRRR-peptide-induced platelet shape change was abolished by a p160^{ROCK} inhibitor, Y27632. These data indicate that at 10 μM concentrations the TFRRR-peptide selectively activates G12/13 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 activates only G12/13 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, causes platelet shape change that is totally blocked by p160^{ROCK} inhibitor Y27632, indicating that such low concentrations of thrombin selectively activates only G12/13

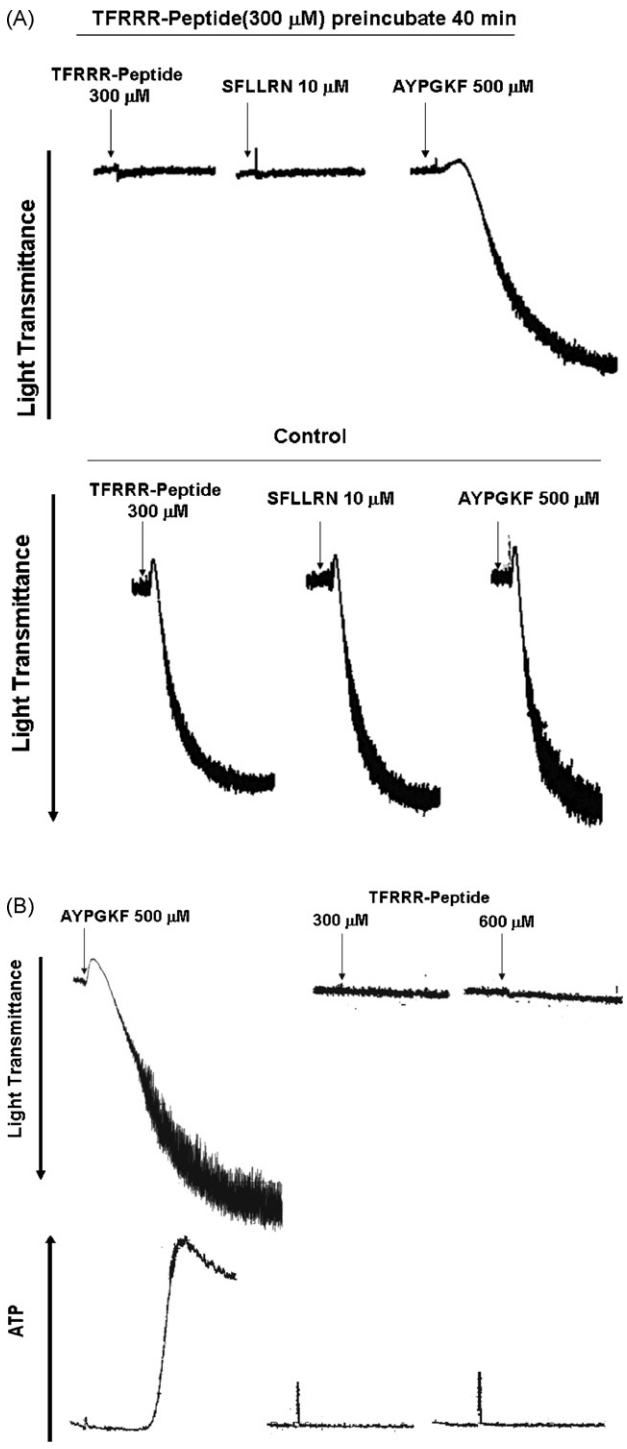

Fig. 3 – The role of PAR-1 in the TFRRR-peptide-induced platelet activation. Washed human platelets were incubated at 37 °C for 40 min in the absence or presence of 300 μM peptide (panel A), followed by stimulation with TFRRR-peptide (300 μM), SFLLRN (10 μM) or AYPGKF (500 μM) to initiate aggregation 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 μM) and TFRRR-peptide (300 and 600 μM) 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.

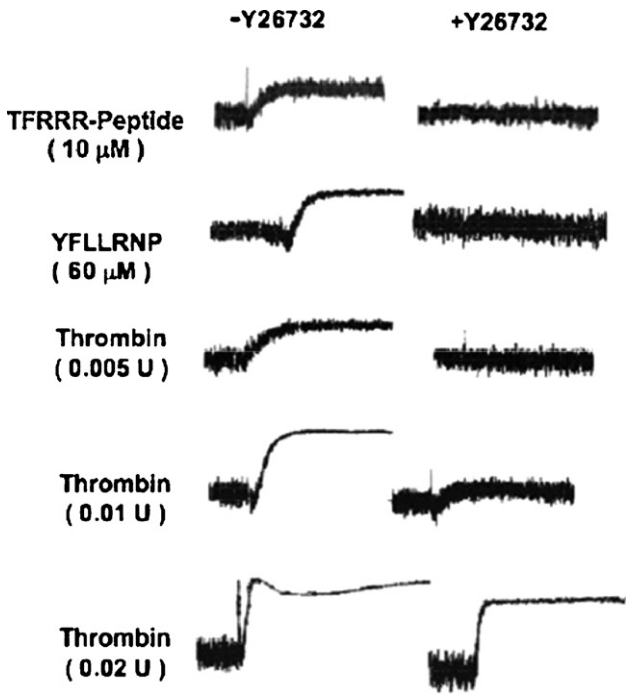

Fig. 4 – Low concentration of TFRRR-peptide or thrombin causes G12/13 pathway activation. Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (10 μM), YFLLRNP (60 μM), or different concentrations of thrombin in the presence and absence of Y27632 (10 μM) under stirring conditions at 37 °C. Tracings were representative of at least three separate experiments.

pathways. As the concentration of thrombin is 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 $P2Y_{12}$ receptor contributes to the irreversible aggregation by SFLLRN [28]. We evaluated the role of $P2Y_{12}$ receptor in TFRRR-induced platelet irreversible aggregation, using AR-C69931MX, a $P2Y_{12}$ 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

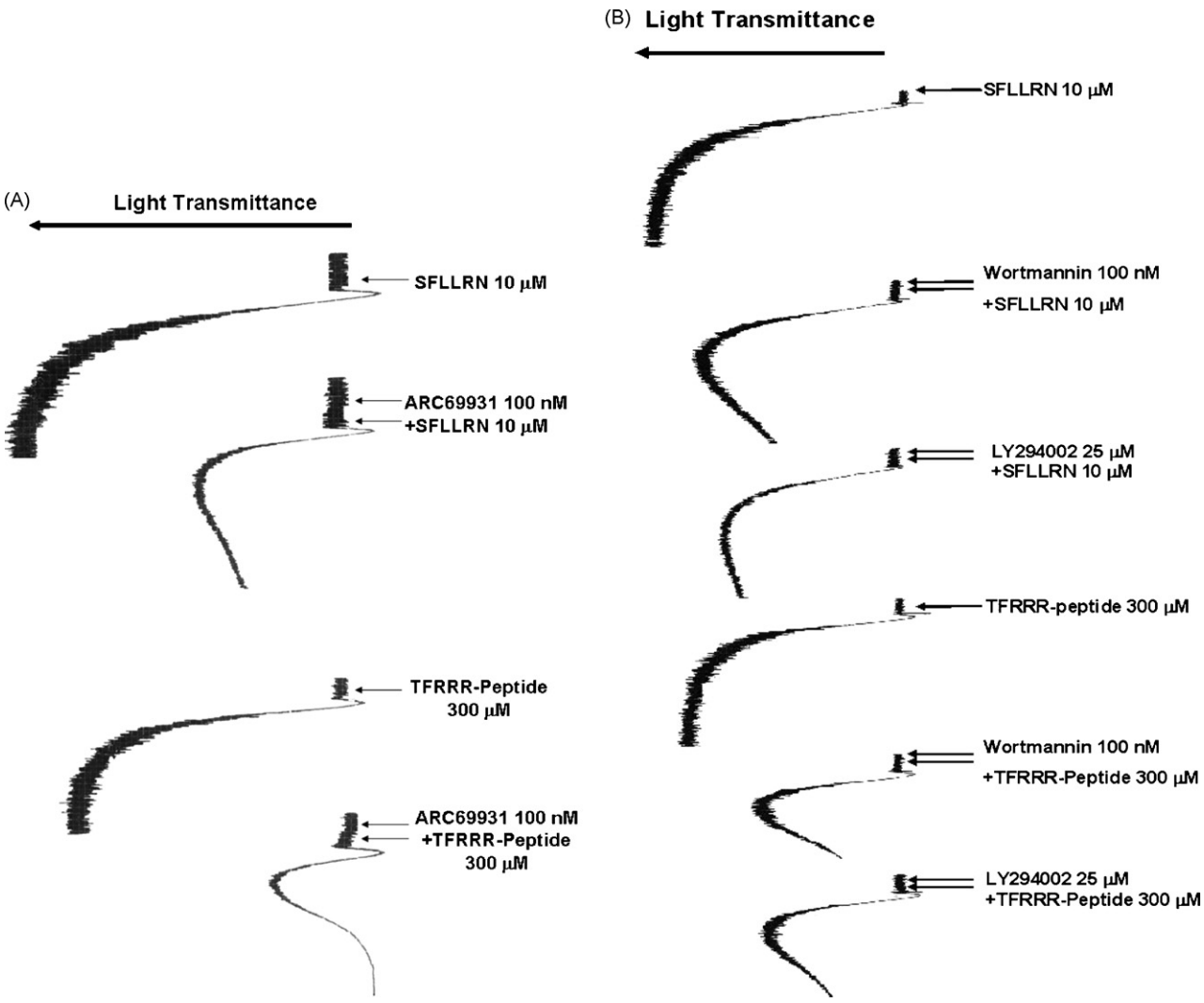

Fig. 5 – Role of secreted ADP and PI3-K in the PAR-1 activating peptide induced platelet irreversible aggregation. Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (300 μM) and SFLLRN (10 μM) in the presence or absence of AR-C69931MX (100 nM) (panel A), wortmannin (100 nM) and LY294002 (25 μM) (panel B) under stirring conditions at 37 °C. Tracings were representative of at least three separate experiments.

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 $P2Y_{12}$ 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 $P2Y_{12}$ receptor [31]. Hence we evaluated the role of $P2Y_{12}$ 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 $P2Y_{12}$ receptor (Fig. 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. Fig. 7 shows that scrambled sequence RTARSLRRRFT failed to cause shape change or aggregation at 300 μM, 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

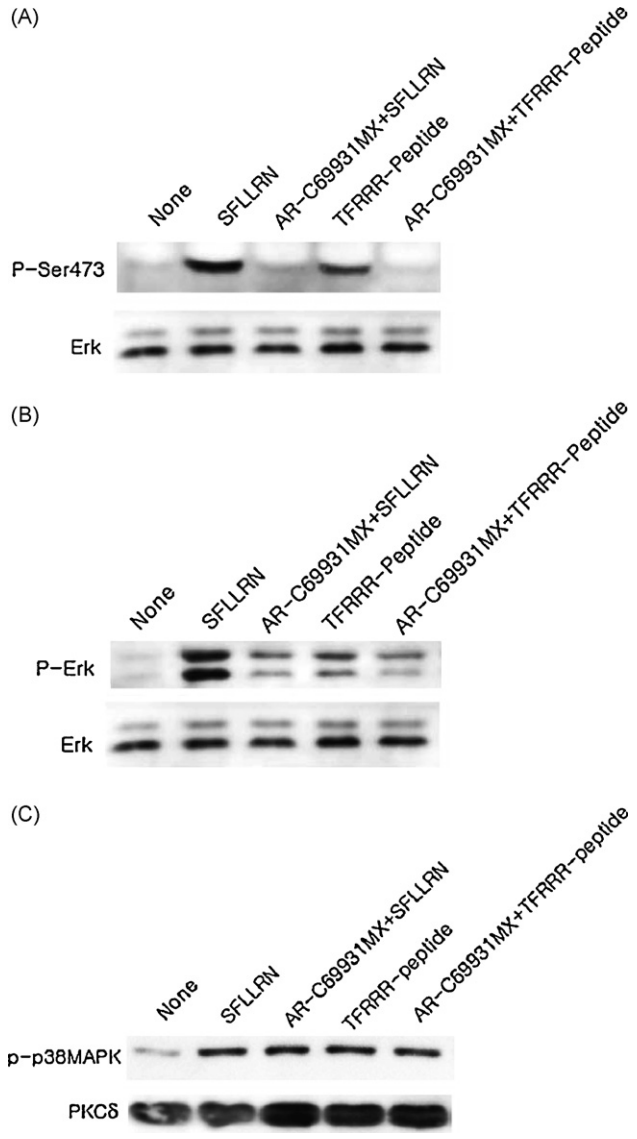

Fig. 6 – Role of $P2Y_{12}$ receptor in TFRRR-peptide induced phosphorylation of Akt, Erk and p38MAPK. Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (300 μM) or SFLLRN (10 μM) for 3 min, in the presence or absence of $P2Y_{12}$ antagonist AR-C69931MX (100 nM). Akt phosphorylation (panel A), Erk phosphorylation (panel B) and p-38 MAPK phosphorylation (panel C) were measured by Western blot analysis. The data are representative of experiments done using platelets from at least three different donors.

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 depends 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.

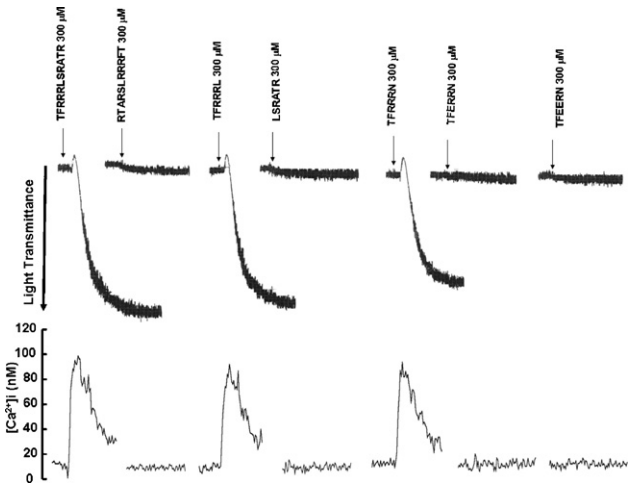

Fig. 7 – Structure–function studies on TFRRR-peptide: Washed aspirin-treated human platelets were stimulated with different peptides derived from the TFRRR-peptide under stirring conditions at 37 °C. In panel A, platelet aggregation and in panel B calcium release were analyzed. (TFRRR-peptide: TFRRRLSRATR; 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.

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 $P2Y_1$ and Gi-coupled $P2Y_{12}$ 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 $P2Y_1$ 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 $P2Y_1$-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 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 μM 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 μM 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 interactions of ligand residue Phe^{43} and LBS-1 residue Ser^{89}, and the hydrophobic contact of LBS-1 residue lle^{88} and ligand residue Leu^{44} are crucial for the ligand binding and receptor activation. Mutation any of these residues will cause dramatically decrease of the receptor activation induced by peptide agonists, whereas the N-terminal of the ligand Ser^{42} is not required for agonist activity and does not make a 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 of PAR-1 whereas SFLLRN is a full agonist.

The peptide 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 Ile^{88} 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 Leu^{44} 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 Leu^{44} 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 in 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 R^{46} (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).

REFERENCES

[1] Jurk K, Kehrel BE. Platelets: physiology and biochemistry. Semin Thromb Hemost 2005;31(4):381–92.

[2] Kahner BN, Shankar H, Murugappan S, Prasad GL, Kunapuli SP. Nucleotide receptor signaling in platelets. J Thromb Haemost 2006;4(11):2317–26.
[3] Shattil SJ, Kashiwagi H, Pampori N. Integrin signaling: the platelet paradigm. Blood 1998;91(8):2645–57.
[4] Brass LF, Manning DR, Cichowski K, Abrams CS. Signaling through G proteins in platelets: to the integrins and beyond. Thromb Haemost 1997;78(1):581–9.
[5] Offermanns S. Activation of platelet function through G protein-coupled receptors. Circ Res 2006;99(12):1293–304.
[6] De Clerck FF, Janssen PA. Amplification mechanisms in platelet activation and arterial thrombosis. J Hypertens Suppl 1990;8(7):S87–93.
[7] Packham MA, Kinlough-Rathbone RL, Mustard JF. Thromboxane A2 causes feedback amplification involving extensive thromboxane A2 formation on close contact of human platelets in media with a low concentration of ionized calcium. Blood 1987;70(3):647–51.
[8] Coughlin SR. How the protease thrombin talks to cells. Proc Natl Acad Sci U S A 1999;96(20):11023–7.
[9] Coughlin SR. Thrombin signalling and protease-activated receptors. Nature 2000;407(6801):258–64.
[10] Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 1991;64(6):1057–68.
[11] Rasmussen UB, Vouret-Craviari V, Jallat S, Schlesinger Y, Pages G, Pavirani A, et al. cDNA cloning and expression of a hamster alpha-thrombin receptor coupled to Ca^{2+} mobilization. FEBS Lett 1991;288(1–2):123–8.
[12] McComsey DF, Hecker LR, Andrade-Gordon P, Addo MF, Maryanoff BE. Macrocyclic hexapeptide analogues of the thrombin receptor (PAR-1) activation motif SFLLRN. Bioorg Med Chem Lett 1999;9(2):255–60.
[13] Scarborough RM, Naughton MA, Teng W, Hung DT, Rose J, Vu TK, et al. Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. J Biol Chem 1992;267(19):13146–9.
[14] Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest 1999;103(6):879–87.
[15] Kahn ML, Zheng YW, Huang W, Bigornia V, Zeng D, Moff S, et al. A dual thrombin receptor system for platelet activation. Nature 1998;394(6694):690–4.
[16] Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. PAR3 is a cofactor for PAR4 activation by thrombin. Nature 2000;404(6778):609–13.
[17] Hung DT, Vu TH, Nelken NA, Coughlin SR. Thrombin-induced events in non-platelet cells are mediated by the unique proteolytic mechanism established for the cloned platelet thrombin receptor. J Cell Biol 1992;116(3):827–32.
[18] Rasmussen UB, Gachet C, Schlesinger Y, Hanau D, Ohlmann P, Van Obberghen-Schilling E, et al. A peptide ligand of the human thrombin receptor antagonizes alpha-thrombin and partially activates platelets. J Biol Chem 1993;268(19):14322–8.
[19] Bauer M, Retzer M, Wilde JI, Maschberger P, Essler M, Aepfelbacher M, et al. Dichotomous regulation of myosin phosphorylation and shape change by Rho-kinase and calcium in intact human platelets. Blood 1999;94(5):1665–72.
[20] Dorsam RT, Kim S, Jin J, Kunapuli SP. Coordinated signaling through both G12/13 and G(i) pathways is sufficient to activate GPIIb/IIIa in human platelets. J Biol Chem 2002;277(49):47588–95.
[21] Shankar H, Kahner BN, Prabhakar J, Lakhani P, Kim S, Kunapuli SP. G-protein-gated inwardly rectifying potassium channels regulate ADP-induced cPLA2 activity in platelets through Src family kinases. Blood 2006;108(9):3027–34.
[22] Quinton TM, Kim S, Dangelmaier C, Dorsam RT, Jin J, Daniel JL, et al. Protein kinase C- and calcium-regulated pathways independently synergize with Gi pathways in agonist-induced fibrinogen receptor activation. Biochem J 2002;368(Pt 2):535–43.
[23] Dubois C, Steiner B, Kieffer N, Reigner SC. Thrombin binding to GPIbalpha induces platelet aggregation and fibrin clot retraction supported by resting alphaIIbbeta3 interaction with polymerized fibrin. Thromb Haemost 2003;89(5):853–65.
[24] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985;260(6):3440–50.
[25] Shankar H, Murugappan S, Kim S, Jin J, Ding Z, Wickman K, et al. Role of G protein-gated inwardly rectifying potassium channels in P2Y12 receptor-mediated platelet functional responses. Blood 2004;104(5):1335–43.
[26] Ding Z, Tuluc F, Bandivadekar KR, Zhang L, Jin J, Kunapuli SP. Arg333 and Arg334 in the COOH terminus of the human P2Y1 receptor are crucial for Gq coupling. Am J Physiol Cell Physiol 2005;288(3):C559–67.
[27] Offermanns S, Laugwitz KL, Spicher K, Schultz G. G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. Proc Natl Acad Sci U S A 1994;91(2):504–8.
[28] Trumel C, Payrastre B, Plantavid M, Hechler B, Viala C, Presek P, et al. A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. Blood 1999;94(12):4156–65.
[29] Kim S, Jin J, Kunapuli SP. Relative contribution of G-protein-coupled pathways to protease-activated receptor-mediated Akt phosphorylation in platelets. Blood 2006;107(3):947–54.
[30] Kim S, Jin J, Kunapuli SP. Akt activation in platelets depends on Gi signaling pathways. J Biol Chem 2004;279(6):4186–95.
[31] Shankar H, Garcia A, Prabhakar J, Kim S, Kunapuli SP. P2Y12 receptor-mediated potentiation of thrombin-induced thromboxane A2 generation in platelets occurs through regulation of Erk1/2 activation. J Thromb Haemost 2006;4(3):638–47.
[32] Shankar H, Kahner B, Kunapuli SP. G-protein dependent platelet signaling–perspectives for therapy. Curr Drug Targets 2006;7(10):1253–63.
[33] Ruggeri ZM. Platelets in atherothrombosis. Nat Med 2002;8(11):1227–34.
[34] Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. J Thromb Haemost 2005;3(8):1800–14.
[35] Gachet C. Regulation of platelet functions by P2 receptors. Annu Rev Pharmacol Toxicol 2006;46:277–300.
[36] Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. Pharmacol Rev 2001;53(2):245–82.
[37] Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci U S A 1998;95(14):8070–4.
[38] Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. J Biol Chem 1998;273(4):2024–9.
[39] Kuliopulos A, Covic L. Blocking receptors on the inside: pepducin-based intervention of PAR signaling and thrombosis. Life Sci 2003;74(2-3):255–62.
[40] Covic L, Gresser AL, Talavera J, Swift S, Kuliopulos A. Activation and inhibition of G protein-coupled receptors by

cell-penetrating membrane-tethered peptides. Proc Natl Acad Sci U S A 2002;99(2):643–8.
[41] Seeley S, Covic L, Jacques SL, Sudmeier J, Baleja JD, Kuliopulos A. Structural basis for thrombin activation of a protease-activated receptor: inhibition of intramolecular liganding. Chem Biol 2003;10(11):1033–41.
[42] Bernatowicz MS, Klimas CE, Hartl KS, Peluso M, Allegretto NJ, Seiler SM. Development of potent thrombin receptor antagonist peptides. J Med Chem 1996;39(25):4879–87.
[43] Vassallo Jr RR, Kieber-Emmons T, Cichowski K, Brass LF. Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. J Biol Chem 1992;267(9):6081–5.
[44] Connolly TM, Condra C, Feng DM, Cook JJ, Stranieri MT, Reilly CF, et al. Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. Thromb Haemost 1994;72(4):627–33.
[45] Hoogerwerf WA, Hellmich HL, Micci MA, Winston JH, Zou L, Pasricha PJ. Molecular cloning of the rat proteinase-activated receptor 4 (PAR4). BMC Mol Biol 2002;3:2.
[46] Ceruso MA, McComsey DF, Leo GC, Andrade-Gordon P, Addo MF, Scarborough RM, et al. Thrombin receptor-activating peptides (TRAPs): investigation of bioactive conformations via structure-activity, spectroscopic, and computational studies. Bioorg Med Chem 1999;7(11):2353–71.
[47] Zhong C, Hayzer DJ, Corson MA, Runge MS. Molecular cloning of the rat vascular smooth muscle thrombin receptor. Evidence for in vitro regulation by basic fibroblast growth factor. J Biol Chem 1992;267(24):16975–9.
[48] Seiler SM, Bernatowicz MS. Peptide-derived protease-activated receptor-1 (PAR-1) antagonists. Curr Med Chem Cardiovasc Hematol Agents 2003;1(1):1–11.