ELSEVIER

European Journal of Pharmacology 321 (1997) 129–135

ejp

Characterization of in vitro and in vivo platelet responses to thrombin and thrombin receptor-activating peptides in guinea pigs

Peter J.S. Chiu *, Glen G. Tetzloff, Carolyn Foster, Madhu Chintala, Edmund J. Sybertz

Department of Pharmacology (2600), Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033-0530, USA

Received 1 August 1996; revised 18 November 1996; accepted 22 November 1996

Abstract

Guinea pig platelets are similar to human platelets in their responsiveness to thrombin receptor-activating peptides and other agonists. Therefore, guinea pigs anesthetized with Inactin (90 mg/kg i.p.) were used to assess in vivo activities of thrombin and thrombin receptor-activating peptides (TRAPs) using ^{111}In-labeled platelets and a microcomputer-based system. The aggregatory responses are expressed as percent change for a 20 min period over basal radioactivity (AUC). Reversible accumulation of platelets occurred in the pulmonary microcirculation in response to stimuli. Human thrombin (50 and 100 U/kg i.v.) caused a dose-related platelet accumulation. Responses of similar magnitude were induced by SFLLRN (TRAP-(1–6)) and Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr-NH_2 (high-affinity thrombin receptor-activating peptide, 0.03, 0.1 and 0.3 mg/kg i.v.). High-affinity thrombin receptor-activating peptide, a new synthetic oligopeptide agonist, is about 3-fold more potent than TRAP-(1–6), a wild-type sequence. Similarly, high-affinity thrombin receptor-activating peptide is about 4 times more potent than TRAP-(1–6) in the radioligand binding study using platelet membrane. By comparison, high-affinity thrombin receptor-activating peptide manifested an aggregatory activity (EC_{60} = 1.2 μM) about 15 times more potent than that of TRAP-(1–6) (EC_{60} = 18.6 μM) in washed guinea pig platelets. The intrapulmonary platelet aggregation in response to thrombin, TRAP-(1–6) and high-affinity thrombin receptor-activating peptide was characterized by long duration (~ 30 min); a reduction in response (18–54%) tended to occur with repeated challenges, presumably due to desensitization and consumption. The response to thrombin (100 U/kg) was greatly inhibited by (D)-Phe-Pro-Arg-chloromethyl ketone (PPACK), a potent thrombin inhibitor (250 μg/kg + 6 μg/kg per min i.v. × 30): AUC, 150 ± 552 vs. 7171 ± 1052 in the control period (n = 8, $P < 0.05$). The response to high-affinity thrombin receptor-activating peptide (0.03 mg/kg), which acts on thrombin receptor directly, was not affected by PPACK. It is concluded that guinea pigs are an appropriate preparation for evaluation of in vivo activity of thrombin inhibitors as well as thrombin receptor agonists and antagonists.

Keywords: Thrombin; Thrombin receptor-activating peptide; Thrombin inhibitor; Platelet aggregation; ^{111}Indium oxine; Receptor binding; Pulmonary microcirculation

1. Introduction

Thrombin is a potent stimulus of platelets, causing shape change, aggregation and secretion from dense granules, α granules and lysosomes (Blockmans et al., 1995). Thrombin receptors from human platelets were cloned and sequenced by Vu et al. (1991). Thrombin receptors are activated by thrombin cleavage generating a new N-terminus. The newly generated SFLLR-containing 'tethered ligand' then activates the G-protein-coupled receptor. Thrombin binds to its receptor through the anion-binding exosite on the enzyme molecule; a portion of the receptor exo-domain resembles the C-terminal of hirudin. Thrombin inhibitors that bind to the active site and/or anion-binding exosite of α-thrombin prevent cleavage of the thrombin receptor to generate tethered ligand.

Thrombin receptor-activating peptides (TRAPs) including SFLLRN (TRAP-(1–6)) and other 'tethered-ligand-derived analogs' directly activate platelets, bypassing the need to generate the receptor-tethered ligand via proteolytic cleavage as is with thrombin (Natarajan et al., 1995; Seiler et al., 1996), e.g., SFLLRN, a 6-mer that represents the wild-type sequence. Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr-NH_2 (high-affinity thrombin receptor-activating peptide), a new synthetic oligopeptide agonist, has greatly enhanced

* Corresponding author. Tel.: (1-908) 298-3236; Fax: (1-908) 298-3294.

PII S0014-2999(96)00931-4

agonist potency with an EC_{50} of 0.01 μM (vs. 0.18 μM for TRAP-(1–6)) in platelet aggregation assay (Feng et al., 1995).

(D)-Phe-Pro-Arg-chloromethyl ketone (PPACK), which irreversibly alkylates the active site histidine 57 of thrombin, is a potent and selective thrombin inhibitor (Weitz et al., 1990; Kaiser and Hauptmann, 1992; Tapparelli et al., 1993). PPACK demonstrates great inhibition of thrombus formation in a number of animal models of arterial and venous thrombosis (Hanson and Harker, 1988; Klement et al., 1992; Schumacher et al., 1993). In this study, PPACK was used as a tool to differentiate the mode of action by which thrombin and thrombin receptor-activating peptides activate platelets.

The platelets of guinea pigs compared to those of rats, rabbits, and dogs resemble more closely human platelets in their responsiveness to thrombin receptor-activating peptides and other agonists (Connolly et al., 1994; Derian et al., 1995). For this reason the guinea pig was chosen to evaluate activities of thrombin and thrombin receptor-derived peptides in vitro using radioligand binding assay in platelet membranes and aggregation of washed platelets and in vivo by measuring intrapulmonary platelet aggregation (Chiu and Tetzloff, 1994; Sandoli et al., 1994).

2. Materials and methods

2.1. Animals

The experiments were carried out in accordance to the National Institutes of Health Guide to the Care and Use of Laboratory Animals and the Animal Welfare Act in a program accredited by the American Association for Accreditation of Laboratory Animal Care. Male Dunkin Hartley guinea pigs (300–550 g) were used (Charles River Breeding Labs., Wilmington, DE, USA).

2.2. Drugs

(D)-Phe-Pro-Arg-chloromethyl ketone, 2 HCl ($FPRCH_2Cl$, PPACK, molecular weight 524.2 (Calbiochem, La Jolla, CA, USA)); the 6 amino acid thrombin receptor-activating peptides, Ser-Phe-Leu-Leu-Arg-Asn-NH_2 (TRAP-(1–6)) and Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr-NH_2 (high-affinity thrombin receptor-activating peptide), were synthesized and supplied by AnaSpec (San Jose, CA, USA); human thrombin (Enzyme Res. Lab., South Bend, IN, USA), 1000 IU/ml stock solution stored frozen in aliquots, diluted with saline; prostaglindin E_1 (Sigma, St. Louis, MO, USA) 1 mg/ml in ethanol (pH 6.5). [^{3}H]High-affinity thrombin receptor-activating peptide was synthesized by Amersham Life Sci. (Arlington Heights, IL, USA).

2.3. Radioligand binding study

2.3.1. Platelet membrane preparation

Donor guinea pigs are anesthetized with Inactin (90 mg/kg i.p.). The abdominal aorta was exposed and entered with a 21-gauge Vacutainer multiple-sample needle and 10–15 ml blood was collected in citrated Vacutainer tubes containing 85 mM sodium citrate, 65 mM citric acid. The guinea pigs were killed by cervical dislocation. Pooled blood was centrifuged for 10 min at $350 \times g$ to obtain platelet-rich plasma. Platelets were then pelleted at $800 \times g$ for 10 min and washed once in 150 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl (pH 7.5). Platelets were resuspended in 5 mM EDTA, 5 mM Tris-HCl (pH 7.5) and disrupted on ice with 20 strokes using a Dounce homogenizer. Membranes were pelleted at 16500 rpm in the SA600 rotor for 20 min and washed once in 20 mM Tris-HCl (pH 7.5) and 1 mM EDTA, suspended in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 1% dimethyl sulfoxide (DMSO), and frozen in liquid nitrogen. Membranes were stored at −80°C until use.

2.3.2. High-volume binding assay

The assay was performed in 96-well Nunc plates (Nunc, Naperville, IL, USA) at a final assay volume of 200 μl (Ahn et al., submitted). Platelet membranes and [^{3}H]high-affinity thrombin receptor-activating peptide were diluted to 400 μg/ml and 25 nM respectively in binding buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.1% bovine serum albumin). DMSO and peptide (10 mM in DMSO) solutions were diluted in binding buffer containing 20% DMSO. Unless otherwise indicated, 20 μl of the above diluted solution of DMSO or peptide and 80 μl of ligand was added to each well and the reaction was started by the addition of 100 μl of membranes. The plates were covered and vortex-mixed gently on a Lab Line Titer Plate Shaker (Lab Line Instruments, Melrose, IL, USA) for 1 h at room temperature. Packard Unifilter GF/C filter plates were soaked for at least 1 h in 0.1% polyethyleneimine. Inclusion of 0.1% bovine serum albumin in the incubation buffer as well as presoaking of the filter plate in 0.1% polyethyleneimine were necessary to reduce binding of [^{3}H]high-affinity thrombin receptor-activating peptide to tubes, pipette tips and the filter plate. The incubated membranes were harvested using the Packard FilterMate Universal Harvester (Packard Instrument, Meriden, CT, USA) and were rapidly washed 4 times with 300 μl ice-cold 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA. MicroScint 20 scintillation cocktail (25 μl) was added to each well, and the plates were counted in the Packard TopCount Microplate Scintillation Counter. The specific binding was defined as the total binding minus the nonspecific binding observed in the presence of excess (50 μM) unlabeled high-affinity thrombin receptor-activating peptide. IC_{50} determinations were performed using at least 5–6 concentrations of peptide. Each point was performed in quadruplicate. IC_{50} values were calculated by the Prism

curve fitting program (GraphPad Software, San Diego, CA, USA).

2.4. Platelet aggregation in vitro

2.4.1. Platelet isolation

Guinea pigs were prepared as in Section 2.3.1 and 15 ml blood was collected in citrated Vacutainer tubes containing 0.38% sodium citrate. The blood was centrifuged at $168 \times g$ for 15 min at 15°C and the platelet-rich plasma obtained was made up to 20 ml with Hepes buffer (pH 7.4) containing prostaglandin E_1 (0.3 μl of a 1 mg/ml stock solution per ml buffer). The Hepes buffer consists of: Hepes (10.0 mM), NaCl (130 mM), KCl (4.74 mM), EGTA (0.2 mM), bovine serum albumin (0.2%) and D-glucose (11.5 mM). The washed platelet suspensions were centrifuged at $750 \times g$ for 7.5 min at 15°C. The platelets were washed again as described above and the pellet was then resuspended in the suspension buffer containing NaCl (130 mM), KCl (4.74 mM), KH_2PO_4 (1.2 mM), $NaHCO_3$ (4 mM), D-glucose (11.5 mM), bovine serum albumin (0.2%), Hepes (10 mM), $MgCl_2 \cdot 6H_2O$ (1.2 mM) and $CaCl_2 \cdot H_2O$ (1.8 mM) and platelet counts were normalized to 300000/μl. Human fibrinogen (400 μg/ml) was added to the final platelet suspension and the platelets were stored at 4°C for 1 h prior to use in the aggregation experiments.

2.4.2. Aggregation assay

Aggregation was performed in a Chronolog dual-channel aggregometer. Briefly, thrombin inhibitors in a volume of 10–20 μl were added to 0.48 ml of platelet suspension and incubated at 37°C for 5 min. Aggregation was initiated by addition of thrombin or thrombin receptor-activating peptides to the platelet suspension in the cuvettes and the aggregation response was then monitored for 5 min on an IBM computer and the peak aggregation response was determined turbidimetrically with the help of the Agglink software (Chronolog). EC_{60} is defined as the concentration of individual agonists that induces a 60% of maximal aggregation response (100% transmittance).

2.5. Intrapulmonary platelet aggregation

2.5.1. Preparation of ^{111}In-labeled platelets

Platelet-rich plasma was obtained by centrifugation at $130 \times g$ for 15 min and made up to 10 ml with Ca^{2+}-free Tyrode's solution containing citrate (10%, v/v) and prostaglandin E_1 (0.3 μg/ml). The diluted platelet-rich plasma was centrifuged at $750 \times g$ for 7.5 min. The pellet was resuspended with Ca^{2+}-free Tyrode's solution and recentrifuged for 7.5 min. The pellet was resuspended in 3 ml of Ca^{2+}-free Tyrode's solution and warmed to 37°C; 6.25 μCi of ^{111}indium oxine (Amersham) for each recipient guinea pig was added and incubated for 3 min. The labeled platelets were pelleted and washed twice with Ca^{2+}-free Tyrode's solution and resuspended in 0.9% saline (approximately 10^8 per ml); the total volume was adjusted so that the labeled platelets were administered in a volume of 0.5 ml to each recipient animal.

2.5.2. Monitoring of intrapulmonary accumulation of ^{111}In-labeled platelets

Guinea pigs, fasted overnight, were anesthetized with Inactin 90 mg/kg i.p.; the trachea and the left jugular vein were cannulated and the animals were placed in a supine position. Intravenous infusion of saline at 2 ml/h was instituted. Washed ^{111}In-labeled platelet suspension (2 μCi in 0.5 ml) was injected via the jugular vein. A sodium iodide crystal scintillation detector was placed immediately above the thorax. A Quad scintillation pre-amplifier/amplifier and a multiplexer/router (Nucleus, Oak Ridge, TN, USA) were used to amplify and select signals from each detector, respectively. Pulses were logged by a processor within a Dell computer, and count rates were displayed graphically (Collimation, Fairfield, NJ, USA).

Aggregatory responses to agonists were determined by summating successive 30-s counts starting 2 min before the challenge and continuing for an additional 20 min after the challenge. All injections were completed with 10 s. Data were transformed to percent change from pre-challenge counts. Area under the curve (AUC) of the '% change vs. time' curve (Figs. 4–6) was estimated by a linear trapezoidal method to obtain aggregatory responses to individual agonists. Washout periods of 30 min separated subsequent challenges. The thrombin inhibitor PPACK was given by a single bolus followed by i.v. infusion for 30 min starting at 10 min before a second aggregatory challenge was administered. Aggregatory responses are expressed as percent change for a 20 min period over basal radioactivity ($AUC_{0-20\ min}$).

2.6. Statistical analysis

All except the radioligand binding data are expressed as mean ± S.E.M. The data were analyzed by analysis of variance (ANOVA) and Bonferroni/Dunn test for comparison between control and different treatment groups. A significance level of 5% was chosen.

3. Results

3.1. Radioligand binding study

In the guinea pig platelet membrane competition binding using [^{3}H]high-affinity thrombin receptor-activating peptide as the radioligand, the IC_{50} values for TRAP-(1–6) and high-affinity thrombin receptor-activating peptide are 75.5 nM (55, 96 nM) and 20.5 nM (21, 20 nM), respectively ($n = 2$), demonstrating that high-affinity thrombin

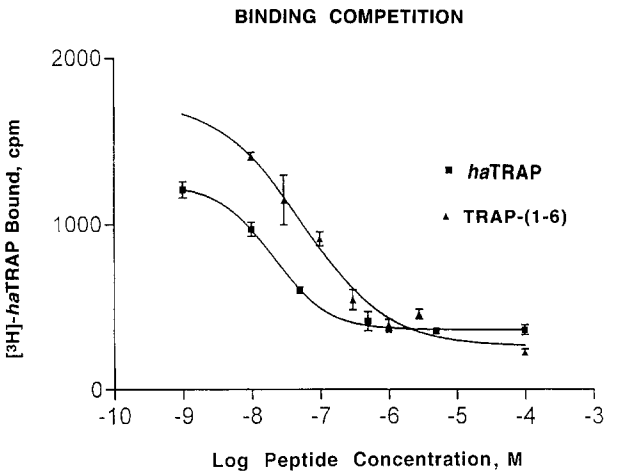

Fig. 1. Effect of TRAP-(1–6) and high-affinity thrombin receptor-activating peptide on [^{3}H]high-affinity thrombin receptor-activating peptide binding to guinea pig platelet membranes. The assay was performed in 96-well Nunc plates at a final assay volume of 200 μl. Platelet membranes and [^{3}H]high-affinity thrombin receptor-activating peptide were diluted to 400 μg/ml and 25 nM respectively in binding buffer. Triangles, TRAP-(1–6); rectangles, high-affinity thrombin receptor-activating peptide (*ha*TRAP). Each point represents mean ± S.D. of 4 observations. The IC_{50} values for TRAP-(1–6) and *ha*TRAP are 55 and 21 nM, respectively.

receptor-activating peptide is 3.7 times more potent than TRAP-(1–6). The results from one binding competition study are illustrated in Fig. 1.

3.2. Platelet aggregation in vitro

TRAP-(1–6) (10, 30 and 100 μM) and high-affinity thrombin receptor-activating peptide (1, 3, and 10 μM) each caused a concentration-dependent aggregation (Fig.

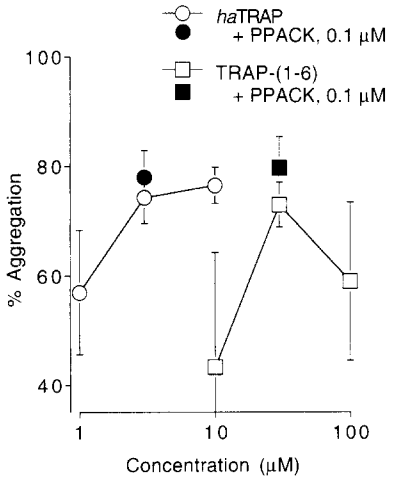

Fig. 2. Aggregatory responses of washed guinea pig platelets to thrombin receptor-activating peptides. Open symbols, TRAP-(1–6) or high-affinity thrombin receptor-activating peptide (*ha*TRAP) alone; closed symbols, TRAP-(1–6) or high-affinity thrombin receptor-activating peptide in the presence of (D)-Phe-Pro-Arg-chloromethyl ketone (PPACK), 0.1 μM. Each point represents mean ± S.E.M. of 3–4 observations.

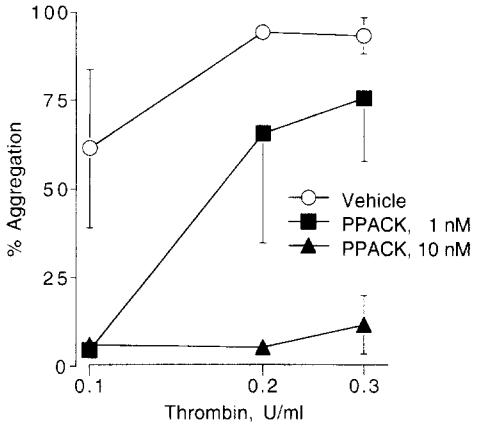

Fig. 3. Inhibition by PPACK of human thrombin-induced aggregatory responses in washed guinea pig platelets. Open symbols, thrombin alone; closed symbols, thrombin (0.1, 0.2 and 0.3 U/ml) in the presence of PPACK (1 and 10 nM). Each point represents mean ± S.E. of 2–4 observations. Some S.E.M. values are too small to be shown on the graph.

2). The EC_{60} for high-affinity thrombin receptor-activating peptide is 1.2 μM, about 15 times that of TRAP-(1–6) (18.6 μM). (D)-Phe-Pro-Arg-chloromethyl ketone (PPACK) at 0.1 μM did not affect the aggregatory response to both TRAP-(1–6) (30 μM) and high-affinity thrombin receptor-activating peptide (3 μM). Thrombin (0.1, 0.2 and 0.3 U/ml) caused pronounced aggregation

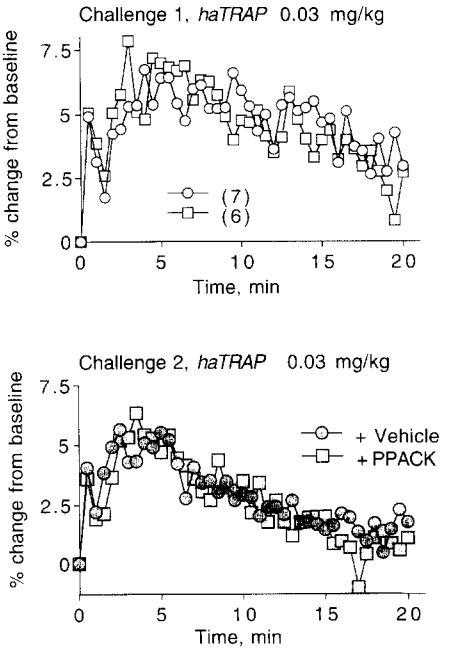

Fig. 4. The time-course of changes in intrapulmonary platelet accumulation in response to high-affinity thrombin receptor-activating peptide in guinea pigs. Top panel, platelet responses to first challenge of high-affinity thrombin receptor-activating peptide (*ha*TRAP) in the basal period; bottom, platelet responses to second challenge which was in the presence of PPACK (250 μg/kg+6 μg/kg per min). Each point represents average of 6–7 observations (cf., Table 1).

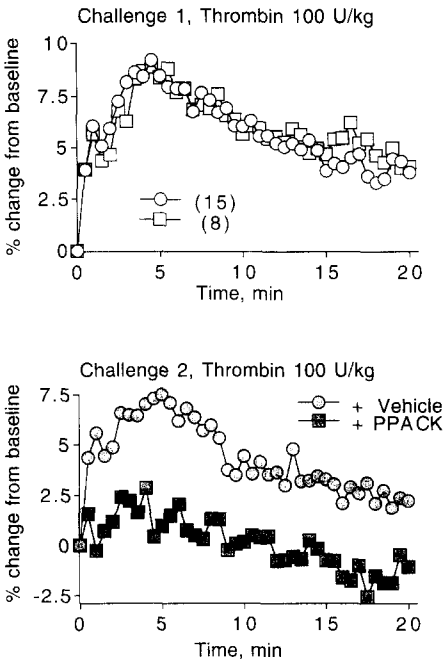

Fig. 5. The time-course of changes in intrapulmonary platelet accumulation in response to human thrombin in guinea pigs. Top panel, platelet responses to first challenge of thrombin in the basal period; bottom panel, platelet responses to second challenge which was in the presence of PPACK (250 μg/kg + 6 μg/kg per min). Each point represents average of 8–15 observations (cf., Table 2).

(Fig. 3); the response to thrombin at 0.1 U/ml, but not at higher concentrations, was completely abolished by PPACK at 1 nM. Moreover, in contrast to thrombin receptor-activating peptides, thrombin at 0.2 and 0.3 U/ml,

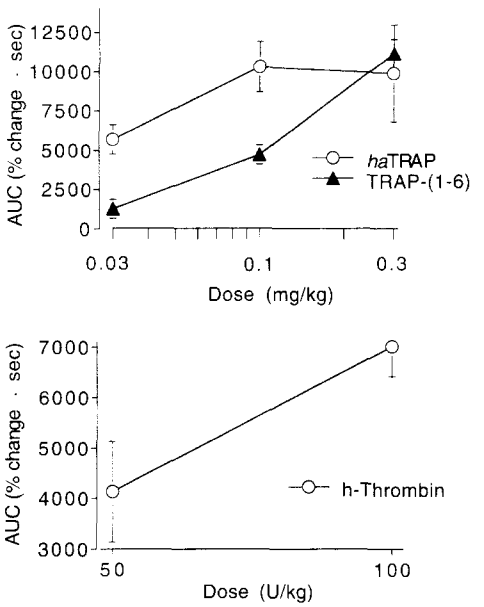

Fig. 6. Dose–response curves for intrapulmonary platelet accumulation induced by high-affinity thrombin receptor-activating peptide, TRAP-(1–6) and thrombin. Top panel, high-affinity thrombin receptor-activating peptide (*ha*TRAP) and TRAP-(1–6); bottom panel, human thrombin. Each point represents mean ± S.E.M. of 4–12 $AUC_{0-20\ min}$ values obtained from the first agonist challenge in individual animals.

Table 1
Intrapulmonary ^{111}In-labeled-platelet aggregation in response to high-affinity thrombin receptor-activating peptide in guinea pigs with or without thrombin inhibition by (D)-Phe-Pro-Arg-chloromethyl ketone (PPACK)

Treatment	*n*	Response ($AUC_{0-20\ min}$)		
		Before	After	Difference
Saline vehicle 2 ml/h	7	5712 ± 926	3470 ± 486	−2242 ± 826
PPACK 250 μg/kg + 6 μg/kg per min i.v. × 30	6	5545 ± 416	3228 ± 646	−2317 ± 802

All values represent mean ± S.E.M. The first (before) high-affinity thrombin receptor-activating peptide challenge (0.03 mg/kg i.v.) was followed by drug treatment with i.v. infusion for 30 min before the second (after) agonist challenge. The aggregatory responses are expressed as percent change for a 20-min period over basal radioactivity (AUC, area under the curve; see Section 2). Cf., Fig. 4.

which induced maximal aggregation, was completely inhibited by PPACK at a concentration as low as 10 nM.

3.3. Intrapulmonary platelet aggregation

High-affinity thrombin receptor-activating peptide (0.03–0.3 mg/kg i.v.) induced a dose-related platelet aggregation, with a time course of action similar to thrombin (50 and 100 U/kg i.v.) which was reversible but took 20–30 min to return to baseline (Figs. 4–6). High-affinity thrombin receptor-activating peptide at 0.1 mg/kg attained a maximal effect. TRAP-(1–6) in the same dose range also caused a dose-dependent increase in platelet response. The magnitude of responses produced by TRAP-(1–6) at 0.03 and 0.1 mg/kg were 3-fold less than those by high-affinity thrombin receptor-activating peptide at the same doses. Animals became cyanotic following i.v. administration of the three high doses consequent to platelet sequestration known to occur in the pulmonary microcirculation with aggregants. As a result, no higher doses were attempted. The magnitute of platelet response to the stimuli also decreased by 18–54% upon repeated challenge (Tables 1 and 2; Figs. 4 and 5). Nonetheless, the responses induced

Table 2
Effects of (D)-Phe-Pro-Arg-chloromethyl ketone (PPACK) on thrombin-induced intrapulmonary ^{111}In-labeled-platelet aggregation in guinea pigs

Treatment	*n*	Response ($AUC_{0-20\ min}$)		
		Before	After	Difference
Saline vehicle 2 ml/h	15	7018 ±	5965250 ± 624	−1769 ± 629
PPACK 125 μg/kg + 2 μg/kg per min i.v. × 30	8	4225 ±	9641495 ± 859 [a]	−2730 ± 1568
PPACK 250 μg/kg + 6 μg/kg per min i.v. × 30	8	7171 ± 1052	150 ± 552 [a]	−7021 ± 1111 [a]

All values represent mean ± S.E.M. The first (before) thrombin challenge (100 U/kg i.v.) was followed by drug treatment with i.v. infusion for 30 min before the second (after) thrombin challenge. The aggregatory responses are expressed as percent change for a 20-min period over basal radioactivity (AUC, area under the curve; see Section 2). Cf., Fig. 5.
[a] $P < 0.05$ vs. corresponding vehicle control, analysis of variance (ANOVA) and Bonferroni/Dunn test.

by high-affinity thrombin receptor-activating peptide (0.03 mg/kg i.v.) were not altered by PPACK (250 μg/kg + 6 μg/kg per min × 30) (Table 1 and Fig. 4). By comparison, PPACK caused a dose-related attenuation of the thrombin-induced intrapulmonary platelet aggregation: −71% at 125 μg/kg + 2 μg/kg per min × 30 and −97% at 250 μg/kg + 6 μg/kg per min × 30, respectively when compared to corresponding vehicle-treated control (Table 2 and Fig. 5).

4. Discussion

Thrombin and thrombin receptor-derived peptide agonists produced reversible and pronounced platelet aggregation in the guinea pig pulmonary microcirculation in a dose-dependent manner. This in vivo assay can differentiate receptor-derived peptide agonists of differing potency. The platelet response to thrombin receptor-derived peptide agonists is insensitive to the thrombin active-site inhibitor (D)-Phe-Pro-Arg-chloromethyl ketone (PPACK), indicating a direct and specific receptor-mediated action. In contrast, the thrombin-induced response is abolished by PPACK as expected. Similarly, Seiler et al. (1996) demonstrated that the human α-thrombin- but not SFLLRNP-stimulated GTPase was inhibited by the thrombin active-site inhibitor GYKI-14766 or the thrombin exosite inhibitor BMS-180742 in CHRF-288 cell membranes.

Guinea pig platelets are known to show sensitivity to thrombin, thrombin receptor-derived peptide agonists and other agonists (thromboxane analogs and platelet-activating factor) more closely similar to human and monkey platelets than platelets from rats and other species (Hirata et al., 1993; Connolly et al., 1994). Guinea pigs responded to thrombin with a robust and prolonged intrapulmonary platelet accumulation which took 20–30 min to return to baseline after challenge, in sharp contrast to the ready reversibility of rat platelet responses (5-min duration) (Chiu and Tetzloff, 1994). Thrombin receptor-activating peptides evoked a similar platelet response in guinea pigs but not in rats (Chintala et al., 1995; Chiu et al., 1995). Another feature of the guinea pig intrapulmonary platelet response to the stimuli is its proclivity to decrease with repeated challenges, which differs sharply from the great reproducibility to repeated agonist stimulation over several hours shown by rats (Oyekan and Botting, 1990; Chiu and Tetzloff, 1994). Probably guinea pig platelets are more prone to desensitization and consumption than rat platelets to account for the difference.

High-affinity thrombin receptor-activating peptide consistently showed a greater activity than TRAP-(1–6) in various assays. We first demonstrated specific binding of thrombin receptor-activating peptides in the isolated guinea pig platelet membranes in which high-affinity thrombin receptor-activating peptide is about 4 times more potent than TRAP-(1–6), similar to the result with human platelet membranes (Ahn et al., 1997). In comparison, high-affinity thrombin receptor-activating peptide is about 15 times more potent than TRAP-(1–6) in the washed guinea pig platelets. The reason for the greatly enhanced potency of high-affinity thrombin receptor-activating peptide relative to TRAP-(1–6) in washed platelets is not clear. However, a similar relative potency for the two thrombin receptor-activating peptides was previously reported in human platelets (Feng et al., 1995). In light of the observation that high-affinity thrombin receptor-activating peptide is about 3 times more potent than TRAP-(1–6) in the guinea pig intrapulmonary platelet aggregation assay, the radioligand binding assay apppears to afford a more accurate prediction of in vivo activity of agonists than the washed platelet aggregation assay.

Thrombin receptor-activating peptides act exclusively through the seven-transmembrane-domain moderate-affinity thrombin receptor (Vu et al., 1991). Most of the effects of thrombin on the human platelets (e.g., aggregation and membrane GTPase stimulation) and on the endothelium (e.g. prostacyclin production) are also elicited by receptor-derived peptides, suggesting that thrombin mainly exerts its effects via interaction between proteolytically generated receptor-tethered ligand and its receptor rather than directly through its proteolytic activity as in blood coagulation (Seiler et al., 1996). Most recently Connolly et al. (1996) reported that adult mice with disruption of the thrombin receptor (*tr*) gene are not associated with bleeding diathesis. Furthermore, $tr^{-/-}$ platelets exhibit a strong response to thrombin, suggesting the existence of a second platelet thrombin receptor subtype in this species. A different thrombin receptor or separate signalling pathways have been presumed to form the basis of species-selective sensitivity to thrombin receptor-activating peptides. Nonetheless, the observations in human platelets that thrombin receptor-activating peptides cause robust aggregation and that antibodies (e.g., anti-TR^{34-52} that prevents cleavage of the TR^{34-52} or anti-$TR^{52-69+Cys}$ that is directed against an 18-amino acid peptide from the hirudin-like domain) or uncleavable receptor-mimicking peptides effectively inhibit thrombin-induced aggregation or secretion support an important role of the cloned thrombin receptor for human platelet function (Hung et al., 1992; Bahou et al., 1993; Norton et al., 1993; Connolly et al., 1994; Derian et al., 1995).

In view of the pivotal role of thrombin in platelet aggregation, blockade of the platelet thrombin receptor with antagonists that do not affect fibrin formation seems a viable approach to prevent arterial thrombosis (Brass, 1995). This is suggested by the finding that blockade of the platelet thrombin receptor with immunoglobulin (Ig) G 9600, a polyclonal antibody raised against a peptide derived from the thrombin-binding exosite region of the cloned human thrombin receptor, effectively inhibited platelet-dependent cyclic flow reductions in the carotid artery of the African green monkey (Cook et al., 1995).

However, the antagonists reported so far are peptides of low potency (Giesberts et al., 1995; Seiler et al., 1995). The peptide YFLLRNP inhibited platelet aggregation and stimulation of platelet membrane GTPase in response to thrombin or SFLLRNP in concentrations of 100 μM and greater (Rasmussen et al., 1993; Giesberts et al., 1995; Seiler et al., 1996); no detectable in vivo receptor antagonist activity has yet been reported. Moreover, the peptide has partial agonist activity. Potent compounds have yet to emerge and manifest significant in vivo efficacy. Based on the characteristics of in vitro and in vivo responses to thrombin and thrombin receptor-activating peptides demonstrated in the present study, it is concluded that the guinea pig intrapulmonary platelet aggregation model is suitable for testing in vivo activities of thrombin inhibitors and both thrombin receptor agonists and antagonists.

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