Rabbit and Rat Platelets Do Not Respond to Thrombin Receptor Peptides That Activate Human Platelets

By Raelene L. Kinlough-Rathbone, Margaret L. Rand, and Marian A. Packham

Human platelets are aggregated and induced to release their granule contents and form thromboxane by peptides as short as 6-amino acid residues (SFLLRN) corresponding to the newly released N-terminus of the thrombin receptor that is cleaved by thrombin. Using washed platelets, we found that these responses to SFLLRN (2 to 6 pmol/L) were enhanced by fibrinogen. However, neither SFLLRN nor SFLLRNPNPDKYEPF had any effect on washed rabbit or rat platelets, although they were fully responsive to human thrombin. Concentrations of the peptides as high as 100 µmol/L did not cause the platelets of rabbits or rats to change shape, aggregate, release granule contents, or form thromboxane. SFLLRN did not affect the extent of aggregation induced by adenosine diphosphate (ADP) or a low concentration of thrombin. Pig platelets responded to 50 µmol/L SFLLRN with reversible aggregation, which was enhanced by fibrinogen, but not accompanied by the release of dense granule contents. Guinea pig platelets aggregated and released granule contents in response to 25 or 50 µmol/L of SFLLRN, but responded with only shape change to lower concentrations. Thus, these experiments indicate that rabbit and rat platelets lack a functional response to human thrombin receptor peptides that fully activate the previously described human thrombin receptor, despite a full response of both rabbit and rat platelets to human thrombin, and that pig and guinea pig platelets have incomplete responses to these human thrombin receptor peptides. The results suggest that platelets of rabbits and rats, and perhaps guinea pigs and pigs, respond to thrombin through an alternative receptor that has also been suggested to be present on human platelets.

MATERIALS AND METHODS

Blood was obtained from New Zealand white rabbits, Wistar rats, and albino Hartley guinea pigs. Pig blood was obtained from a slaughterhouse. Human or rabbit platelets were isolated, labeled with 3H-serotonin (and in some experiments with 51Cr), and suspended in Tyrode-albumin solution as previously described.8 Rabbit platelets and guinea pig platelets in citrated platelet-rich plasma (one part 3.8% trisodium citrate to nine parts of blood) were also prepared. Suspensions of rat platelets were prepared by the method used for rabbit platelets; suspensions of pig platelets were prepared as described for human platelets, except that the osmolarity of the washing and suspending media was adjusted to 340 mOsm/L. The final suspending medium was Tyrode’s solution containing 5 mMol/L HEPES, 0.35% albumin, and apyrase; platelet counts were adjusted to 500,000/mL. In some experiments, human fibrinogen (AB Kabi, Stockholm, Sweden) was added to the suspending medium. Shape change and aggregation were studied turbidimetrically11; 100% aggregation was defined as the difference in light transmission through a platelet suspension and the suspending medium. Release of 3H-serotonin and loss of 51Cr were measured as described elsewhere.5,12 Thromboxane B2 (TXB2) formation was assayed with radioimmunoassay kits from NEN (Lachine, Quebec, Canada). SFLLRNPNPDKYEPF, a gift from Dr. S.C.-T. Lam, was prepared by solid-phase synthesis in the Laboratory of the Research Resources Center of the University of Illinois at Chicago, IL; SFLLRN was synthesized by the Institute for Molecular Biology and Biotechnology (MOBIX) at McMaster University, Hamilton, Ontario, Canada. Human thrombin (T 6759) was from Sigma Chemical, St Louis, MO.

RESULTS

Human platelets. As reported by other investigators,1,3,7,8 human platelets changed shape, aggregated, released dense granule contents, and formed thromboxane in response to both peptides. The presence of fibrinogen (400 µg/mL) in the suspending medium increased the aggregation response and the percentage of 3H-serotonin released from human platelets (Fig 1A and B); in the presence of fibrinogen, concentrations less than 6 µmol/L SFLLRN caused maximum aggregation (Fig 1B). There was some variation in the responsiveness of platelets from different donors. As noted by Chao et al,1 who studied human platelets in platelet-rich plasma, a small increase in the concentration of the peptide changed the aggregation response from only shape change to maximum aggregation. A similar observation was made with thrombin-induced aggregation and release of 3H-serotonin (Fig 1C). With low concentrations of SFLLRN, aggregation appeared to be reversible and the shape of the aggregation curves resembled that of primary adenosine diphosphate (ADP)-induced aggregation.13 Loss of 51Cr was less than 1% in association with maximum aggregation induced by SFLLRN or thrombin, indicating

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that lysis was not occurring. In two experiments performed to degranulate human platelets, 100 μmol/L SFLLRN-PNDKYEPF (in the absence of fibrinogen) caused 91% and 92% release of 14C-serotonin. In similar experiments, SFLLRN (20 μmol/L) caused 95% ± 2.1% release of 14C-serotonin and formation of 409 ± 209 ng of TXB2 per 10⁶ platelets (means ± SEM; n = 4).

Rabbit and rat platelets. Unlike the findings with human platelets, neither peptide had any apparent effect on rabbit or rat platelets in either the presence or absence of fibrinogen. In seven separate experiments, suspensions of washed platelets from rabbits did not change shape, aggregate, or release 14C-serotonin in response to 50 or 100 μmol/L SFLLRN or 10 to 100 μmol/L SFLLRN (Fig 2A). No formation of TXB2 by rabbit platelets was detectable in response to 100 μmol/L SFLLRN. Similarly, concentrations of SFLLRN as high as 120 μmol/L had no effect on rabbit platelets in citrated platelet-rich plasma.

Fig 2. (A) Lack of response of rabbit platelets to SFLLRN (10 to 100 μmol/L) in the presence of 400 μg/ml fibrinogen (Fbg; there was also no response in the absence of fibrinogen); responses to thrombin are also shown: (a) 0.05 U/mL, (b) 0.075 U/mL, and (c) 0.1 U/mL. (B) Responses of pig platelets to SFLLRN (50 μmol/L) in the presence and absence of fibrinogen, and to thrombin (1 U/mL). (C) Responses of guinea pig platelets in citrated platelet-rich plasma to SFLLRN: (a) 10 μmol/L, (b) 25 μmol/L, and (c) 50 μmol/L (typical of three experiments). The percentages of 14C-serotonin released in 3 minutes are shown in the boxes beside the aggregation curves.

Suspensions of rabbit platelets aggregated and released 14C-serotonin (20 to 80%) in response to low concentrations (0.05 to 0.1 U/mL) of human thrombin (Fig 2A). SFLLRN (100 μmol/L) did not affect the extent of aggregation, release of 14C-serotonin, or TXB2 formation induced by 0.02 to 0.1 U/mL of thrombin. This peptide also did not affect the extent of aggregation of rabbit platelets caused by 1 μmol/L ADP.

Lysis, assessed with 51Cr-labeled platelets, was not detectable in response to the highest concentrations of SFLLRN that were tested with suspensions of rabbit platelets.

Even in the presence of fibrinogen, rat platelets were also completely unresponsive to both peptides at concentrations ranging from 10 to 100 μmol/L. SFLLRN (75 μmol/L) caused no release of 14C-serotonin or formation of TXB2. However, rat platelets aggregated strongly (70% aggregation) in response to 0.1 or 0.2 U/mL of thrombin. They released 35% of their serotonin on aggregation with 0.2 U/
Pig platelets. The effect of SFLLRN was also tested with pig platelets. Concentrations as high as 50 μmol/L did not cause the release of 14C-serotonin, although they caused reversible aggregation; the extent of this aggregation response was increased by the presence of fibrinogen (Fig 2B). SFLLRN-induced aggregation occurred without a lag phase with a pattern similar to the reversible aggregation response caused by 5 or 10 μmol/L ADP in the presence of fibrinogen (results not shown). Pig platelets were responsive to human thrombin after a lag phase, although higher concentrations were required than with human, rabbit, or rat platelets (Fig 2B).

Guinea pig platelets. Guinea pig platelets in citrated platelet-rich plasma aggregated in response to 25 or 50 μmol/L SFLLRN, but only shape change occurred at lower concentrations (10 and 15 μmol/L) (Fig 2C). Release of 14C-serotonin induced by 50 μmol/L SFLLRN averaged 44% (three experiments). The guinea pig platelets aggregated strongly to ADP and released 37% of their 14C-serotonin, indicating that secondary aggregation occurred.

DISCUSSION

In accord with the observations of other investigators,\(^1^7\) we found that both SFLLRN and SFLLRNPNNDKYEPF caused human platelet shape change, aggregation, release of dense granule contents, and TXB\(_2\) formation. Platelets from different donors varied in their responsiveness, but maximum aggregation was achieved at concentrations of SFLLRN less than 6 μmol/L. The presence of fibrinogen in the suspending medium increased the extent of aggregation and release of 14C-serotonin, indicating that released fibrinogen was insufficient to support peptide-induced aggregation to the fullest extent. In contrast, rabbit and rat platelets did not respond in any way to the peptides in concentrations as high as 100 μmol/L. Particularly noteworthy is the absence of even a shape change response of rabbit and rat platelets in concentrations (IO and 15 μmol/L) (Fig 2C).

Guinea pig platelets in citrated platelet-rich plasma aggregated in response to 25 or 50 μmol/L SFLLRN, but only shape change occurred at lower concentrations (10 and 15 μmol/L) (Fig 2C). Release of 14C-serotonin induced by 50 μmol/L SFLLRN averaged 44% (three experiments). The guinea pig platelets aggregated strongly to ADP and released 37% of their 14C-serotonin, indicating that secondary aggregation occurred.

Although we are not aware that the sequences of the rabbit, rat, guinea pig, or pig platelet thrombin receptors have been studied, sequences for the thrombin receptor of human, hamster and mouse have been obtained.\(^1\)\(^3\)\(^4\)\(^7\) The regions surrounding the cleavage site of the receptor are almost identical, particularly in the domains considered to be essential for receptor activation. The peptide corresponding to SFLLRN of the human receptor is SFLLRN in the hamster and mouse. Vouret-Craviari et al\(^9\) have shown that the hamster peptide causes the release of serotonin from human platelets, and Vassallo et al\(^1\) reported that SFALRN aggregated human platelets with the concentration required for half-maximal aggregation being 2 μmol/L, compared with 0.8 μmol/L for SFLLRN. Chao et al\(^7\) also reported that substitution of alanine for phenylalanine in the third position of the 14-residue peptide (SFLLRNPNNDKYEPF) resulted in an extent of aggregation of human platelets that was similar to that caused by SFLLRN. None of the peptides appears to have been tested in vitro with platelets from species other than human.

However, the human peptide SFLLRN has been shown to cause intravascular platelet aggregation when administered intravenously to guinea pigs.\(^5\) In keeping with these findings, we observed aggregation of guinea pig platelets in vitro by SFLLRN, albeit with higher concentrations than were required with human platelets.

One would expect the rabbit and rat peptide(s) to be more similar to the hamster and mouse peptide than to the human. If the rabbit and rat peptides are the same as the hamster peptide, it seems unlikely that the Phe-Leu difference in the third position of the peptides would account for the lack of effect of high concentrations of the human peptide on rabbit and rat platelets. Nevertheless, the possibility that differences in the amino acid compositions of the peptides are responsible for our findings cannot be ruled out. Although Chao et al\(^7\) found that the region of the hamster peptide between residues 7 and 14 was detrimental to agonist activity with human platelets, our observations that high concentrations of both SFLLRNPNNDKYEPF and SFLLRN were without effect on rabbit and rat platelets indicate that species differences in the peptides between residues 7 and 14 are not responsible for these observations, since SFLLRN does not contain these possibly detrimental peptides.

The lack of response of rabbit and rat platelets to high concentrations of SFLLRNPNNDKYEPF and SFLLRN is surprising, since rabbit and rat platelets are fully responsive to human thrombin in the same concentration range as human platelets. Possible explanations include lack of the thrombin receptor identified by Vu et al,\(^1\) an inactivating mutation at the site where the agonist peptide binds to the receptor, the lack of, or a defect in, transmembrane signaling by the receptor, or a species difference in downstream signaling.

Coughlin et al\(^1\) have pointed out that thrombin responses that are not mimicked by the agonist peptide may be mediated by a different mechanism. They have suggested the possibility that platelets may have thrombin receptors in addition to that cloned originally by Vu et al.\(^1\) Reports by other investigators provide strong indications of the existence of at least two thrombin receptors on human platelets.\(^1\)\(^5\)\(^17\) Seiler et al\(^17\) obtained evidence for two pathways of thrombin-induced activation of human platelets by examining the ability of the 14-amino acid thrombin receptor-activating peptide to desensitize platelets to thrombin; they found that platelets were desensitized to low concentrations of α-thrombin, but not to γ-thrombin. This group also studied a thrombin exosite inhibitor that inhibited platelet aggregation induced by low, but not high concentrations of α-thrombin. Seiler et al\(^17\) reviewed the literature concerning two pathways, pointing out that one pathway, which they termed R\(_1\), appears to be activated by low concentrations of α-thrombin (<0.1 U/mL) and involves a high-affinity
thrombin binding site \((kd = 0.3 \text{ nmol/L})\). This pathway leads to activation of phospholipase \(A_2\), secretion, and inhibition of adenylate cyclase; it does not operate after chymotrypsin treatment of platelets,\(^{15}\) is insensitive to \(\gamma\)-thrombin, and requires continued occupation of the thrombin receptor.\(^{16}\) The other pathway of activation by thrombin, termed \(R_2\) by Seiler et al,\(^{17}\) seems to be activated by higher concentrations of \(\alpha\)-thrombin and to be mediated by a moderate affinity binding site \((kd = 10 \text{ nmol/L})\). They point out that it is through this pathway that thrombin activates phospholipase \(C\) and protein kinase \(C\), and mobilizes calcium, leading to aggregation and release of granule contents. Unlike \(R_1\), this pathway is responsive to \(\gamma\)-thrombin and continual occupation of the receptor by \(\alpha\)-thrombin is not required.\(^{16}\) Further evidence for two receptors for thrombin has been reported recently.\(^{18-20}\) Bahou et al,\(^{18}\) using \(F(ab')_2\) fragments of an antibody to the N-terminal 160 amino acids of the cloned thrombin receptor, found that although aggregation induced by 0.1 U/mL of thrombin was nearly abolished, a delayed shape change response persisted. Greco et al\(^{19}\) pointed out that the cloned thrombin receptor has characteristics similar to the moderate-affinity thrombin receptor. By blocking access to the high-affinity thrombin-binding site on glycoprotein \(Ib\alpha\) with a monoclonal antibody, they showed that both pathways are required for optimal platelet activation by \(\alpha\)-thrombin. Gralnick et al\(^{20}\) came to a similar conclusion from their studies with Bernard-Soulier platelets. In addition, SFLLRN does not involve the moderate affinity receptor cloned by Vu et al,\(^{1}\) but probably involves the alternative receptor, or receptors.

Since high concentrations of SFLLRN neither inhibited nor potentiated the responses of rabbit platelets to human thrombin, it would appear that the peptide does not compete with thrombin for the alternative receptor that is responsible for thrombin-induced responses of rabbit platelets. In addition, SFLLRN does not have nonspecific inhibitory effects on rabbit platelets, since it did not affect ADP-induced aggregation.

The findings that rabbit and rat platelets are unresponsive to these peptides, and that pig and guinea pig platelets are almost an order of magnitude less responsive than human platelets, leads one to speculate that platelets from different species, all of which respond to thrombin, differ in the proportions of the two receptors or their characteristics. Rabbit or rat platelets may be useful for studies of the receptor that is not activated by SFLLRN.

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