INTRODUCTION

Inflammation is a localized protective response to tissue injury. Early in the inflammatory process, neutrophils respond to chemotactic stimuli by adhering to the vascular endothelium and migrating into the inflamed tissue. Transendothelial migration of neutrophils requires the formation of stable adhesions between cell surface molecules and their receptors expressed on neutrophils and endothelial cells. Since inflammation is often associated with a procoagulant environment, thrombin generation is thought to influence these cellular interactions. Thrombin is known to induce a transient, reversible adherence of neutrophils to endothelial cells. Since inflammation is often associated with a procoagulant environment, thrombin generation is thought to influence these cellular interactions. Thrombin is known to induce a transient, reversible adherence of neutrophils to endothelial cells. Since inflammation is often associated with a procoagulant environment, thrombin generation is thought to influence these cellular interactions.

The response to thrombin of human neutrophils: evidence for two novel receptors

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SUMMARY

Human α-thrombin was a chemoattractant for human neutrophils yielding a maximal response of similar magnitude to that observed with formyl-Met-Leu-Phe. The observed chemotaxis was not due to stimulation of the proteolytically activated thrombin receptor since: (1) this receptor was not detected by flow cytometry; (2) the inactive thrombin mutant Ser¹⁹⁵→Ala elicited a chemotactic response indistinguishable from that caused by wild-type thrombin; (3) antibodies to the cleavage site of the proteolytically activated receptor did not affect thrombin-induced chemotaxis; (4) a thrombin receptor activating peptide (TRAP) failed to stimulate chemotaxis. These data indicate the existence of a thrombin receptor for neutrophil chemotaxis which is not activated by proteolysis. In addition, although wild-type and Ser¹⁹⁵→Ala thrombin did not cause an increase in intracellular Ca²⁺, a Ca²⁺ response to TRAP was observed with neutrophils from some donors. The TRAP-induced increase in Ca²⁺ was reproducible, dose dependent and specific. The use of alanine-substituted peptides demonstrated that the Ca²⁺ response was due to TRAP stimulation of a receptor other than the proteolytically activated thrombin receptor. Thus, it is necessary to re-evaluate the assumption made in previous studies that responses to TRAP are mediated by the proteolytically activated thrombin receptor.

Key words: thrombin, thrombin receptor, neutrophil, chemotaxis, inflammation

INTRODUCTION

Inflammation is a localized protective response to tissue injury. Early in the inflammatory process, neutrophils respond to chemotactic stimuli by adhering to the vascular endothelium and migrating into the inflamed tissue. Transendothelial migration of neutrophils requires the formation of stable adhesions between cell surface molecules and their receptors expressed on neutrophils and endothelial cells. Since inflammation is often associated with a procoagulant environment, thrombin generation is thought to influence these cellular interactions. Thrombin is known to induce a transient, reversible adherence of neutrophils to endothelial cells (Bizios et al., 1988) through stimulation of endothelial cell P-selectin expression and synthesis of platelet activating factor by the endothelium (Toothill et al., 1990; Lorant et al., 1991). Thrombin-induced endothelial cell ICAM-1 expression appears to mediate the prolonged neutrophil adhesion associated with targeting neutrophils to inflammatory sites (Sugama et al., 1992). Endothelial gap formation is also stimulated by thrombin (Laposta et al., 1983), which could assist in the transmigration of neutrophils from the circulation into the inflamed tissue.

At least some of these effects of thrombin on endothelial cells appear to be mediated by the proteolytically activated thrombin receptor found on platelets. Thrombin activates this receptor by a novel mechanism involving a specific cleavage within the extracellular domain of the receptor. This cleavage generates a new N terminus, which then acts as a tethered ligand for the receptor. A peptide analog of the new N-terminal region, thrombin receptor activating peptide (TRAP), was able to activate the receptor in the absence of thrombin (Vu et al., 1991). TRAP was found to reproduce the effects of thrombin on P-selectin expression, but failed to promote ICAM-1 expression (Sugama et al., 1992). The role of the proteolytically activated thrombin receptor in increasing endothelial permeability is controversial; TRAP appeared to stimulate endothelial cell contraction in one study (Garcia et al., 1993), but was unable to mimic this effect of thrombin in another (Lum et al., 1993). Proteolytically inactive derivatives of thrombin failed to stimulate neutrophil migration across endothelial cell monolayers (Drake and Issekutz, 1992), suggesting a role for a cleavage-dependent thrombin receptor in at least part of this process.

The effect of thrombin on neutrophil migration is not confined to a direct action on the endothelium. In addition, thrombin has been shown to stimulate the chemotaxis of neutrophils (Bizios et al., 1986; Cohen et al., 1991; Morin et al.,...
Three residues (Pro60B-Pro 60C-Trp 60D) were deleted from thrombin to that of Bode et al. (1989) and is based on that of chymotrypsin (Hofsteenge, 1986). Two recombinant thrombin mutants were replaced by alanine to yield the alanine-substituted TRAP series. This was measured using a 48-well micro-chemotaxis chamber apparatus (Neuro Probe, Cabin John, MD, USA). For chemotaxis studies, blood from healthy adult donors was collected in preservative free heparin (10 units/ml), layered onto an equal volume of Ficoll/Isopaque solution and spun at 650 g for 20 minutes. The cell pellet was mixed with an equal volume of 3% Dextran T500 (w/v) (Pharmacia) in phosphate buffered saline and incubated at 37°C for 45 minutes. The top layer containing the neutrophils was spun at 200 g for 10 minutes. The pellet was resuspended in red blood cell lysis buffer (8.275 g NH₄Cl, 1 g NaHCO₃, 0.0372 g EDTA in 1 litre), incubated on ice for 8 minutes and spun at 200 g for 10 minutes. After washing once in Hanks’ balanced salt solution (HBSS), the neutrophils were resuspended at a density of 2.5×10⁵ cells/ml in RPMI 1640 containing 0.5% (w/v) bovine serum albumin (BSA) and 10 mM HEPES buffer (pH 7.2). The chemotactractors or control reagents were placed in each well of the bottom chamber. A polyvinyl pyrolidone-free polycarbonate membrane (3 μM pores, Nucleopore, Pleasanton, CA, USA) was placed over a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) wetted with distilled water. Both membranes were positioned over the wells with the polycarbonate membrane uppermost, and a 50 μl aliquot of neutrophils was added to each well of the top chamber. Following incubation for 30 minutes at 37°C, the polycarbonate membrane was removed and fixed in 95% isopropanol (v/v) for 10 minutes at room temperature. The nitrocellulose was discarded. The upper side was scraped, and the membrane was stained with diamidino-2-phenylindole (2.5 mg/ml). After washing and mounting, the neutrophils were quantified by fluorescence microscopy with computer-assisted image analysis (SeeScan, Cambridge, UK). Cell nuclei from two fields in each well were counted. The data are presented as the average of four wells ± s.e.m. for each concentration of agonist. All experiments were repeated at least twice with similar results.

The stimulation of directional neutrophil movement (chemotaxis) versus random cell movement (chemokinesis) by thrombin, S195A and des-PPW thrombin were compared in order to validate their chemoattractant effect. Each of the thrombins at a concentration of 10⁻⁸ M was placed in both the upper and lower chambers. Under these conditions, the number of cells migrating through the membrane was not greater than that obtained when HBSS alone was present in both chambers. The numbers of cells per field were 40±4, 41±2, 49±4 and 42±3 with HBSS, thrombin, S195A and des-PPW, respectively. Thus, these agonists exerted no chemokinetic effect on neutrophils.

**Measurement of intracellular Ca²⁺ responses in neutrophils**

Neutrophils were isolated from whole blood of male donors using Percoll (Pharmacia) solutions as described by Jepsen and Skottun (1982). Venous blood (50 ml) was collected from healthy volunteers into lithium heparin tubes, diluted 1:1 with normal saline and 5 ml was layered onto a Percoll gradient formed by overlaying 3 ml 78% Percoll with 2 ml 54% Percoll. The tubes were centrifuged at 200 g for 15 minutes. If necessary, the cell pellet was resuspended in ice-cold red blood cell lysis buffer and contaminating red blood cells allowed to lyse on ice for 5 minutes before centrifugation at 4°C. The pellet was resuspended in EM + 0.2% (w/v) BSA, cells were counted and centrifuged at 200 g for 10 minutes. Cells were resuspended at a concentration of 6×10⁶/ml in EM containing 0.2% (w/v) BSA and loaded with Fura-2 at room temperature as described previously (Bootman et al., 1992; Jenkins et al., 1993). Cells were then centrifuged and resuspended in EM at 2.5×10⁶ cells/ml for fluorescence measurements. The concentration of intracellular Ca²⁺ ([Ca²⁺]i) was determined using a Perkin Elmer LS-50 fluorometer. Fura-2 fluorescence was measured at excitation and emission wave-

**MATERIALS AND METHODS**

**Materials**

Human α-thrombin was prepared as previously described (Stone and Hofsteenge, 1986). Two recombinant thrombin mutants were produced as described by Le Bonniec et al. (1993). In the mutant S195A, the active-site serine (Ser¹⁹⁵; the numbering of thrombin is that of Bode et al. (1989) and is based on that of chymotrypsin) was mutated to alanine which resulted in a total loss of catalytic activity. Three residues (Pro₆₀₈-Pro₆₀₉-Trp₆₀₉) were deleted from thrombin to generate the mutant des-PPW (Le Bonniec et al., 1993). Fura-2 acetoxyethyl ester was from Molecular Probes (Eugene, OR, USA). Ficoll/Isopaque, Dextran T500 and Percoll were purchased from Pharmacia (Uppsala, Sweden). The TRAP routinely used in this study consisted of 9 amino acids with the sequence SFLLRNPND. For studies examining the effects of alanine substitution, the peptides were variants of a TRAP of 14 residues with the sequence SFLLRNPND-KYEFPP (Vu et al., 1991); the first six residues were sequentially replaced by alanine to yield the alanine-substituted TRAP series. These peptides were synthesized as previously described (Chao et al., 1992). All other chemicals were of the highest purity available commercially.

**Neutrophil chemotaxis**

This was measured using a 48-well micro-chemotaxis chamber
Purity of neutrophil preparations

To assess the purity of the neutrophil preparations, 1 drop of foetal calf serum followed by 2 drops of cells (1×10⁶ cells/ml) were placed in the chamber of a cytospin apparatus and spun at 25 g for 5 minutes onto a glass slide. Slides were allowed to air dry and stained for morphological identification with Giemsa stain. All preparations contained more than 95% neutrophils with the major contaminant being eosinophils. Contamination of the preparations by platelets was insignificant as assessed by the absence of immunohistochemical staining for CD61.

Data analysis

One way ANOVA was used to analyze the difference between the chemotactic response to reagents versus the HBSS control. The dependence of peak increase in [Ca²⁺] on the concentration of TRAP was fitted to the Hill equation by nonlinear regression to estimate the maximal response, the concentration of agonist causing a half-maximal response (EC₅₀), and the Hill coefficient (n).

RESULTS

Neutrophil chemotaxis

Thrombin induced neutrophil chemotaxis in a dose-dependent manner (Fig. 1A); a response to thrombin was observed with neutrophils from each of the 7 donors tested. Thrombin triggered directional movement of neutrophils at very low concentrations, with a maximal effect at 10⁻⁸ M, while at higher concentrations, cell movement was inhibited. This dose-dependence is similar to that observed in response to other chemotactants such as fMLP, a synthetic formyl-peptide that resembles products of bacterial metabolism, and leukotriene B₄ (Lew, 1989). The maximum response to thrombin was similar to that obtained with 10⁻⁷ M fMLP which under the conditions of the assay with this preparation of fMLP was the optimal con-
centration. Hirudin at a 4-fold molar excess over thrombin was able to block the chemotactic response of neutrophils to $10^{-7}$, $10^{-8}$ and $10^{-9}$ M thrombin. Hirudin alone elicited no chemotactic response (data not shown).

The role of the proteolytically activated thrombin receptor in mediating neutrophil chemotaxis was addressed using three different experimental approaches: the chemotactic responses of neutrophils to TRAP and a mutant of thrombin lacking proteolytic activity (S195A) were evaluated and the ability of antibodies to the cleavage site of the receptor to block thrombin-induced chemotaxis was assessed. TRAP (0.5-500 µM) was found to have no significant effect on neutrophil chemotaxis (Fig. 1B). Dose-dependent chemotactic responses were elicited by S195A thrombin, with a maximal chemotactic effect at $10^{-8}$ M (Fig. 1C); the maximal response to S195A was similar to that observed with thrombin and fMLP. Preincubation of the neutrophils with antibodies to the thrombin cleavage site of the receptor (anti-SFLL) at a concentration (60 µg/ml), which has previously been shown to block the response of platelets and Saos-2 cells to thrombin (Howells et al., 1993; Jenkins et al., 1993), did not affect the chemotactic response to thrombin. After preincubation of the neutrophils for 30 minutes in HBSS alone, the chemotactic responses to HBSS, $10^{-7}$ M fMLP and $10^{-8}$ M thrombin were 39±7, 122±8, and 105±13 cells/field, respectively. Preincubation with 60 µg/ml anti-SFLL (or 60 µg/ml preimmune immunoglobulin G) did not significantly affect the responses which were 47±5 (51±5), 93±7 (107±8) and 126±12 (89±8) cells/field with HBSS, $10^{-7}$ M fMLP and $10^{-8}$ M thrombin, respectively (values obtained after preincubation with preimmune immunoglobulin G are given in brackets). Taken together, these results indicate that the proteolytically activated thrombin receptor is not involved in thrombin-induced neutrophil chemotaxis, since: (1) the proteolytically activated receptor cannot be activated by the S195A mutant of thrombin; (2) it can be activated by TRAP (Vu et al., 1991); and (3) its activation by thrombin is blocked by antibodies against the cleavage site (Brass et al., 1992; Jenkins et al., 1993).

A region of thrombin thought to be responsible for monocyte chemotaxis has been identified (Bar Shavit et al., 1983, 1984). This region is centred around the B-insertion loop, which is not found in other serine proteases. A mutant of thrombin in which three residues were deleted from this loop (Le Bonniec et al., 1993) was found to elicit dose-dependent chemotaxis in neutrophils (Fig. 1D). Since disruption of the B-insertion of thrombin had no significant effect on the chemotactic properties of thrombin on neutrophils, it appears that other motifs comprise the neutrophil chemotactic domain of thrombin.

**Effect of thrombin and S195A thrombin on [Ca$^{2+}$]$i$ in neutrophils**

Since many chemotactic factors elicit an increase in the concentration of intracellular calcium ([Ca$^{2+}$]$i$) in neutrophils (O’Flaherty et al., 1991), the Ca$^{2+}$ signalling response to thrombin and S195A was tested. Thrombin (0.2-200 nM) had no significant effect on [Ca$^{2+}$]$i$ in neutrophils from each of six blood donors (Fig. 2A). This observation is consistent with other studies demonstrating an absence of thrombin-induced Ca$^{2+}$ signalling in human neutrophils (Hoffman and Church, 1993; Takahashi et al., 1992). The inactive thrombin mutant S195A (10-200 nM) also failed to stimulate a rise in [Ca$^{2+}$]$i$ in each of the six donors (Fig. 2B). As a positive control, unpreincubation with 60 M thrombin were 39±7, 122±8, and 105±13 cells/field, respectively. Preincubation with 60 µg/ml anti-SFLL (or 60 µg/ml preimmune immunoglobulin G) did not significantly affect the responses which were 47±5 (51±5), 93±7 (107±8) and 126±12 (89±8) cells/field with HBSS, $10^{-7}$ M fMLP and $10^{-8}$ M thrombin, respectively (values obtained after preincubation with preimmune immunoglobulin G are given in brackets). Taken together, these results indicate that the proteolytically activated thrombin receptor is not involved in thrombin-induced neutrophil chemotaxis, since: (1) the proteolytically activated receptor cannot be activated by the S195A mutant of thrombin; (2) it can be activated by TRAP (Vu et al., 1991); and (3) its activation by thrombin is blocked by antibodies against the cleavage site (Brass et al., 1992; Jenkins et al., 1993).

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Effect of TRAP on \([\text{Ca}^{2+}]_i\) in neutrophils

Neutrophils from the same six donors were also tested for a Ca\(^{2+}\) response to TRAP. Given the absence of a Ca\(^{2+}\) response to thrombin, no response to TRAP was expected. Surprisingly, a Ca\(^{2+}\) response to TRAP was observed with the neutrophils from certain donors. Neutrophils isolated from three out of six individuals failed to respond to TRAP (200–400 μM), while TRAP-stimulated increases in \([\text{Ca}^{2+}]_i\) were observed in neutrophils obtained from the other three donors (Fig. 3A,B). The magnitude of the TRAP response also varied from donor to donor (in Fig. 3B); compare the robust response of donor i to those of donors ii and iii. Despite the variations between donors, the presence or absence of a TRAP response was observed consistently for each donor; donors i and ii exhibited a Ca\(^{2+}\) response of the same magnitude on more than 6 occasions over a period of 12 months. Pretreatment of the neutrophils from donors i and ii with thrombin or S195A (100 nM) did not affect the Ca\(^{2+}\) response of the neutrophils to TRAP (data not shown). The Ca\(^{2+}\) response was specific. A control peptide, in which the order of the first two residues is reversed (FSLLRNPNDKYEPF), fails to activate the proteolytically activated thrombin receptor (Vu et al., 1991). Neutrophils from TRAP-responsive donors did not exhibit a \([\text{Ca}^{2+}]_i\) rise when this control peptide was used (200 μM; Fig. 3C).

The TRAP response could not be attributed to contaminating cells in the purified neutrophil population. Neutrophils constituted 97–99% of the cells in the samples that responded to TRAP. Moreover, platelets were absent from these preparations as determined by immunocytochemical staining using an antibody against the platelet-specific marker CD61.

In TRAP-responsive cells, the magnitude of the \([\text{Ca}^{2+}]_i\), rise observed in response to TRAP was dependent on the concentration of TRAP as shown in Fig. 4 for donor i. Analysis of these data according to the Hill equation yielded estimates of 327±20 nM for the maximal \([\text{Ca}^{2+}]_i\), rise and 72±8 μM for the concentration of TRAP yielding the half-maximal effect (EC\(_{50}\)). The Hill coefficient (n) was 1.9±0.2. The EC\(_{50}\) for TRAP with this neutrophil preparation was within the range of those previously observed for TRAP responses in other cell types.

Fig. 3. Effect of TRAP on \([\text{Ca}^{2+}]_i\) in neutrophils. TRAP-stimulated Ca\(^{2+}\) responses in Fura-2 loaded neutrophils. The trace in A is representative of the response observed with three donors; neutrophils from each donor were tested between 2 and 5 times. The three traces in B (i-iii) show the three TRAP-responsive donors (note the difference in magnitude); similar traces were obtained on at least 5 separate occasions. The arrow indicates the point of addition of 200 μM TRAP to the cells. The axes shown at the bottom of part B give the time and \([\text{Ca}^{2+}]_i\) scales for all three traces. In C, 200 μM FSLLRNPNDKYEPF was added to the cells at the arrow. The trace in C is representative of the response observed with the neutrophils from four donors, two of which responded to TRAP.

Fig. 4. Dose-response relationship of TRAP on \([\text{Ca}^{2+}]_i\) in neutrophils. Concentration-response curve for TRAP-stimulated \([\text{Ca}^{2+}]_i\) elevation in populations of Fura-2 loaded neutrophils (mean ± s.e.m., n=3) isolated from donor i (see Fig. 3). The values represent peak \([\text{Ca}^{2+}]_i\) elevation above the prestimulated level.
Table 1. Effect of alanine-substituted TRAPs on [Ca^{2+}]_{i} in neutrophils

<table>
<thead>
<tr>
<th>Residue substituted</th>
<th>Percentage response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser1'</td>
<td>0*</td>
</tr>
<tr>
<td>Phe2'</td>
<td>0*</td>
</tr>
<tr>
<td>Leu3'</td>
<td>0*</td>
</tr>
<tr>
<td>Leu4'</td>
<td>177±15</td>
</tr>
<tr>
<td>Arg5'</td>
<td>0*</td>
</tr>
<tr>
<td>Asn6'</td>
<td>97±12</td>
</tr>
</tbody>
</table>

Neutrophils were obtained from donor ii, loaded with Fura-2 and challenged with a series of alanine-substituted peptides (200 μM). The activity of the peptides is expressed as a percentage of the peak [Ca^{2+}]_{i} elevation observed with unsubstituted TRAP (mean ± s.e.m.; n = 3). Amino acid residues in TRAP are labelled with primed numbers that indicate their position in the cleaved thrombin receptor. Therefore, Ser1' is equivalent to Ser42 of the deduced amino acid sequence (Vu et al., 1991).

No Ca^{2+} response was observed with these alanine-substituted peptides.

types (Vu et al., 1991; Vuoret-Craviari et al., 1993; Suidan et al., 1992; Joseph and MacDermot, 1993; Garcia et al., 1993).

The lack of a thrombin-induced [Ca^{2+}]_{i} rise in neutrophils indicated that an increase in intracellular [Ca^{2+}]_{i} was not involved in the chemotactic response to thrombin. Moreover, differences between the thrombin and TRAP Ca^{2+} responses raised the possibility that TRAP was activating another receptor. To assess this possibility, the response of neutrophils to a series of alanine-substituted peptides was examined. The relative importance of residues in TRAP for its agonist activity with neutrophils was determined by sequentially replacing each of the first six N-terminal residues by alanine. The results are given in Table 1. The response (peak increase in [Ca^{2+}]_{i}) of neutrophils to the alanine-substituted peptides (200 μM) is expressed as a percentage of that observed with 200 μM TRAP. As shown in Table 1, the amino acids in positions 1, 2, 3, 4, and 5 were essential for activity; no response was observed when these residues were replaced by alanine. Replacing leucine in position 4 increased the activity of the peptide, while replacement of asparagine in position 6 did not affect significantly the neutrophil Ca^{2+} response.

DISCUSSION

The role of thrombin as a link between the inflammatory response and the coagulation cascade has been the subject of much investigation. Thrombin has direct effects on the vascular endothelium which result in the production of several pro-inflammatory substances, including platelet activating factor (Prescott et al., 1984) and PGI2 (Jaffe et al., 1987). Thrombin also stimulates the transendothelial migration of neutrophils into inflamed tissues, by inducing endothelial cell gap formation (Laposta et al., 1983) and mediating adhesive interactions between neutrophils and the endothelium (Bizios et al., 1988). Many of these effects appear to occur, at least in part, via the activation of a platelet-type (proteolytically activated) thrombin receptor.

Enhanced vascular permeability is not likely to be the sole cause for neutrophil migration in response to thrombin, because agonists which increase endothelial cell permeability, such as histamine, cannot reproduce fully the effects of thrombin on the accumulation of neutrophils in inflamed tissues (Drake and Issekutz, 1992). The present work has demonstrated that thrombin has a chemotactic effect on neutrophils, and that this effect is unlikely to be mediated by the platelet-type thrombin receptor. Thrombin fulfils its role as a chemoattractant by stimulating directional, rather than random movement of neutrophils. The dose-dependence of thrombin-induced neutrophil chemotaxis (Fig. 1A) was found to be similar to that of other chemoattractants, which at low concentrations promote chemotaxis, and at high concentrations arrest cell movement and trigger secretion (Lew, 1989). Both thrombin and a mutant thrombin without catalytic activity (S195A) had chemotactic effects on neutrophils (Fig. 1A,C). TRAP, which activates the proteolytically activated thrombin receptor, was found to have no effect on neutrophil chemotaxis (Fig. 1B). These observations extend the results of an earlier study using chemically inactivated thrombin, which also pointed to a non-enzymatic chemoattractant action of thrombin on neutrophils (Bizios et al., 1986). Furthermore, preincubation of neutrophils with polyclonal antibodies raised against the cleavage site of the proteolytically activated receptor failed to inhibit thrombin-induced chemotaxis.

Thus, the results obtained in the present study indicate that a receptor distinct from the proteolytically activated one is responsible for neutrophil chemotaxis. Following coagulation, neutrophils immediately migrate into the thrombus (Henry, 1965). During the inflammatory response that follows, proteases released from neutrophils may cleave thrombin to generate chemotactic, degraded thrombin fragments. Indeed, the results of Bar Shavit et al. (1983, 1984) indicate that a fragment of thrombin which contains the unique B-insertion loop is chemotactic for monocytes. However, it should be noted that other results suggest that the B-insertion loop may not be chemotactic for human monocytes. Joseph and MacDermot (1993) found that the B-insertion loop peptide did not stimulate actin polymerization, which is thought to be a prerequisite for chemotaxis, with a monocytic cell line (U-937). Moreover, TRAP caused actin polymerization with this cell line suggesting that the proteolytically activated thrombin receptor may be responsible for thrombin-induced chemotaxis in monocytes. Whether or not the B-insertion loop plays a role in thrombin-induced monocyte chemotaxis, it is unlikely to be involved in thrombin-induced neutrophil chemotaxis. Disruption of this loop did not attenuate thrombin’s actions on neutrophil chemotaxis (Fig. 1D), suggesting that other regions of thrombin may be involved in the response of neutrophils. The results obtained with hirudin help to delineate slightly the chemotactic domain. Hirudin treatment abolished neutrophil chemotaxis in response to thrombin (Bizios et al., 1986; Morin et al., 1991) indicating that hirudin binding masks thrombin’s chemotactic domain. It has been suggested that the anion-binding exosite forms part of this domain, since C-terminal fragments of hirudin, which bind to this site, are capable of inhibiting thrombin-induced neutrophil chemotaxis (Rowand et al., 1992).

The signalling pathways which mediate neutrophil chemotaxis are poorly understood. Many chemotactic agents, including fMLP, platelet activating factor and leukotriene B4, elicit a rise in [Ca^{2+}]_{i} in neutrophils (O’Flaherty et al., 1991). In contrast, the chemotactic effect of thrombin occurred in the absence of a detectable rise in [Ca^{2+}]_{i}; thrombin-induced [Ca^{2+}]_{i} transients were not observed in neutrophils isolated...
from the peripheral blood of six donors (Fig. 2A). These results indicate that increases in 
$[\text{Ca}^{2+}]_i$ do not play a role in thrombin-induced chemotaxis. The results with thrombin are consistent with the results from a number of other studies which indicate that increases in 
$[\text{Ca}^{2+}]_i$ are not essential for the chemotactic response of neutrophils. There is considerable evidence that a rise in 
$[\text{Ca}^{2+}]_i$ is not a prerequisite for fMLP-induced neutrophil chemotaxis (Elferink et al., 1992; Meshulam et al., 1986; Perez et al., 1989). Furthermore, neutrophil actin polymerization and migration across endothelial cell monolayers in response to fMLP do not appear to require an increase in 
$[\text{Ca}^{2+}]_i$ (Kuijpers et al., 1992). Neutrophils can also degranulate in the absence of appreciable 
$[\text{Ca}^{2+}]_i$ transients after stimulation with a number of receptor agonists (O’Flaherty et al., 1991). Taken together, these studies suggest that a measurable change in 
$[\text{Ca}^{2+}]_i$ is not an absolute requirement for some of the events that follow neutrophil activation. Further studies are required to elucidate the second messenger pathways involved in the chemotactic response of neutrophils to thrombin.

Interestingly, despite the absence of a thrombin 
$[\text{Ca}^{2+}]_i$ transient, neutrophils isolated from some donors exhibited a rise in 
$[\text{Ca}^{2+}]_i$ with TRAP (Fig. 3B). The identity of the neutrophil receptor activated by TRAP is unknown, but our studies indicate that it is not the proteolytically activated thrombin receptor. Firstly, thrombin failed to stimulate a detectable 
$\text{Ca}^{2+}$ response in TRAP-responsive neutrophils (Fig. 2A). Flow cytometric analysis of peripheral blood, using affinity-purified polyclonal antibodies to TRAP, and a purified monoclonal antibody against the extracellular domain of the platelet-type thrombin receptor, demonstrated an absence of thrombin receptor staining in neutrophils (Howells et al., 1993; our unpublished observations). Lastly, the residues in TRAP required for receptor activation of neutrophil 
$\text{Ca}^{2+}$ signalling did not correspond to those necessary for activation of the thrombin receptor in platelets. Using a series of alanine-substituted peptides, the amino acids in positions 1, 2, 3 and 5 in TRAP were shown to be essential for activating the neutrophil receptor, while replacing leucine with alanine in position 4 increased the activity of the peptide (Table 1). These results differ from those obtained in studies examining TRAP stimulation of the proteolytically activated thrombin receptor (Chao et al., 1992; Scarborough et al., 1992; Vassallo et al., 1992) which demonstrated that the side chains of phenylalanine, leucine, and arginine in positions 2, 4 and 5, respectively, were essential for full peptide activity. Thus, the specificity of the neutrophil receptor differs from that of the proteolytically activated thrombin receptor in that thrombin receptor activation requires the side chain of leucine in position 4, but does not require the side chain of serine in position 1. These results suggest that the binding site of the TRAP receptor on neutrophils differs considerably from that of the tethered ligand generated after thrombin cleavage of the thrombin receptor. It is possible that the TRAP response in neutrophils may reflect activation of a receptor closely related to the proteolytically activated thrombin receptor, perhaps belonging to a family of receptors activated by cleavage and tethered ligand binding. In this respect, the recent discovery of a second proteolytically activated receptor (PAR-2) is particularly interesting (Nystedt et al., 1994). Like the proteolytically-activated thrombin receptor, PAR-2 is a G-protein-coupled receptor that can be activated by cleavage within its extracellular domain to create a new N terminus that acts as a tethered ligand. The tethered ligand for PAR-2 shows homology with TRAP (SLIGRL versus SFLLRN) suggesting that both agonist peptides might not be entirely specific (Nystedt et al., 1995). The possibility that PAR-2 is the neutrophil receptor activated by TRAP is currently being investigated. The fact that the neutrophils from only a fraction of the donors exhibited an increase in 
$[\text{Ca}^{2+}]_i$ in response to TRAP suggests that the level of expression of the receptor varies. Alternatively, since TRAP is unlikely to be the natural ligand for the receptor, the ability of the receptor from different individuals to accommodate TRAP may vary.

In conclusion, the results of the present study indicate that the different cellular effects of thrombin are mediated by more than one type of thrombin receptor. In particular, the receptor responsible for neutrophil chemotaxis does not require proteolysis. Identification of this chemotaxis receptor, and elucidation of the signalling pathways it employs, will undoubtedly shed light on the search for other thrombin receptors. In addition, evidence was obtained for a receptor distinct from the proteolytically activated thrombin receptor that is activated by TRAP and couples to increases in intracellular 
$\text{Ca}^{2+}$. It has previously been assumed that if TRAP reproduced the cellular effects of thrombin, then the proteolytically activated thrombin receptor originally described by Vu et al. (1991) was involved. The results obtained in the present study indicate that this assumption is not valid.

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