The N-terminal thrombin receptor fragment SFLLRN, but not catalytically inactive thrombin-derived agonists, activate U937 human monocytc cells: evidence for receptor hydrolysis in thrombin-dependent signalling

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It has previously been reported that murine macrophages can respond chemotactically and mitogenically to the serine proteinase thrombin. There is a similar response in these macrophages to catalytically inactivated thrombin or to peptide fragments of the thrombin B-chain [Bar-Shavit, Kahn, Mann and Wilner (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 976–980]. However, the existence of a non-proteolytic mechanism of thrombin receptor activation in mononuclear cells was not evident in the present study using U937 human monocytc cells. The ability of thrombin to stimulate intracellular Ca2+ mobilization, actin polymerization or cell proliferation was not mimicked by Nα-tosyl-L-lysine chloromethyl ketone (TLCK)-treated thrombin or by a synthetic 14-amino-acid peptide (single amino acid letter code YPPWKNFTENDL) corresponding to a part of the B-chain of thrombin which was reported to be mitogenic in murine macrophages. Evidence was obtained, however, in U937 cells for the presence of a proteolytic-dependent thrombin receptor similar to the thrombin receptor expressed in platelets, which following thrombin cleavage exposes a new N-terminal tethered ligand. In support of this, a thrombin-receptor-derived hexapeptide (TRP; sequence SFLLRN), corresponding to a part of the thrombin receptor tethered ligand, mimicked all the actions of thrombin in U937 cells. Further, TRP and thrombin cross-desensitized U937 cells to subsequent stimulation with either TRP or thrombin, suggesting that TRP acted through the same U937 cell surface receptor as did thrombin. Thrombin activation of U937 monocytc cells can therefore be accounted for entirely by a proteolytic mechanism of thrombin receptor activation.

INTRODUCTION
The serine proteinase thrombin has a central and well-established role in haemostasis and coagulation [1]. A less familiar action of thrombin is its potential role as a pro-inflammatory mediator acting on monocyte/macrophages. Studies have shown that thrombin is both chemotactic and mitogenic for human peripheral blood monocytes and various macrophage cell lines [2–4]. These studies have also shown that the chemotactic and mitogenic effects of thrombin were mediated by catalytically inactive thrombin derivatives such as di-isopropylphosphorofluoridate treated thrombin, Nα-tosyl-L-lysine chloromethyl ketone (TLCK)-treated thrombin or D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK)-treated thrombin [2,3]. Furthermore, peptide fragments of the B-chain of thrombin, which are totally devoid of proteinase activity, were chemotactic and mitogenic [2,3]. Specifically, based on the amino acid sequence data for human prothrombin [5], a synthetic 14-amino-acid peptide corresponding to thrombin B-chain residues 367–380 was mitogenic, while a peptide fragment corresponding to thrombin B-chain residues 338–400 was both chemotactic and mitogenic [3,4].

The studies of Bar-Shavit et al. [2–4] suggest that activation of human blood monocytes and murine macrophages by thrombin occurs via a hormone-like non-proteolytic receptor-dependent mechanism, thus clearly contrasting with the proteolytic mechanism of thrombin receptor activation operating in platelets, endothelial cells and fibroblasts [6–13]. The proteolytic mechanism of thrombin receptor activation was recently elucidated following the cDNA cloning of the thrombin receptor present in DAMI human megakaryoblastic cells and in CCL39 Chinese hamster fibroblasts [6–8]. A unique feature of the cloned thrombin receptor is that receptor activation requires the proteolytic cleavage by thrombin of the N-terminal extracellular domain of the receptor [6–8]. The newly exposed N-terminal sequence then functions as a tethered ligand which binds to an as yet undefined region of the receptor, resulting in receptor activation. In support of this, a 14-amino-acid synthetic peptide corresponding to a part of the newly created N-terminal thrombin receptor sequence (in the amino acid single letter code, SFLLRNPNDKYEPF and SFFLRNPGENTFEL for the human and hamster thrombin receptors respectively) mimics the ability of thrombin to activate DAMI or CCL39 cells [6–9]. In addition, the 14-amino-acid peptide has been shown to activate platelets, endothelial cells, fibroblasts and the megakaryoblastic HEL cell line [6–13]. It follows that thrombin receptor activation in all of these cells requires proteolytic cleavage of the thrombin receptor. More recent structure–activity studies have revealed that the cellular actions of thrombin can be mimicked by a hexapeptide based on the first six residues of the 14-residue thrombin receptor peptide (SFLLRN and SFFLRN for the human and hamster thrombin receptor respectively) [9,12,14].

In the present study we have determined whether the non-proteolytic mechanisms of thrombin receptor activation reported previously in human blood monocytes and murine macrophage cells [2–4] is common to other monocyte/macrophage cell systems. We have used the U937 human leukaemic monocyte/macrophage cell line, a well-characterized cell line known to display many of the features of monocytes/macrophages [15]. U937 cells respond to thrombin stimulation with the activation

Abbreviations used: TLCK, Nα-tosyl-L-lysine chloromethyl ketone (Tos-Lys-CH2Cl); PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; [Ca2+], intracellular free Ca2+ concentration; TRP, thrombin receptor peptide; NBD, 7-nitrobenz-2-oxa-1,3-diazole; FCS, foetal calf serum.
of phospholipase C, the mobilization of intracellular Ca\(^{2+}\), actin polymerization and chemotaxis [2,16,17]. The presence of a non-proteolytic mechanism was assessed using the inactive thrombin derivative TLCK-thrombin. We have also examined in U937 cells the mitogenic effect of the synthetic 14-amino-acid peptide corresponding to thrombin B-chain residues 367–380 (Peptide 1; YPPWKNFTENDLL), which was reported to be mitogenic in various murine macrophage cell lines [3,4].

The human prothrombin protein sequence of Butkowski et al. [5], on which Peptide 1 is based, conflicts with other published human prothrombin protein sequences in its amide assignment [18,19]. In particular, there is an Asn [5] instead of an Asp [18,19] at position 371 in the thrombin B-chain. Consequently, in the present study we have examined the mitogenic effects of a second 14-amino-acid peptide, which is also based on thrombin B-chain residues 367–380, but with substitution of an Asp for Asn at residue 371 (Peptide 2; YPPWKKNFTE-NDLL). We have also addressed the question of whether a proteolytic mechanism of thrombin receptor activation operates in U937 cells using the hexapeptide thrombin receptor peptide (TRP; sequence SFLLRN) [12,14].

Our results indicate that, in U937 human monocytic cells, thrombin stimulates intracellular Ca\(^{2+}\) mobilization, actin polymerization and mitogenesis by a mechanism solely involving the proteolytic cleavage of the thrombin receptor. The actions of thrombin were mimicked by TRP but not by the various proteolytically inactive thrombin B-chain peptides or by TLCK-thrombin.

**MATERIALS AND METHODS**

**Materials**

Bovine thrombin (95 units/mg of protein) was purchased from Sigma. Indo-1/AM and the serine protease inhibitors TLCK and PPACK were purchased from Novabiochem, Nottingham, U.K. 7-Nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin was obtained from Molecular Probes, Eugene, OR, U.S.A. The thrombin-receptor-derived hexapeptides SFLLRN (TRP), SFLLRD and SALLRD, and the thrombin B-chain peptides Peptide 1 (YPPWKNFTENDLL) and Peptide 2 (YPPWKKNFTE-NDLL), were synthesized by the Peptide Laboratory, Haemostasis Department, M.R.C. Clinical Research Centre, Northwick Park Hospital, London, U.K. The purity of the peptides was determined by h.p.l.c. and the identity of each peptide was confirmed by amino acid analysis and mass spectroscopy. [\(^3\)H]Thymidine (17.5 Ci/mmol) was obtained from Amersham. Human α-thrombin (3000 units/mg of protein) was a gift from Professor D. Lane, Charing Cross Medical School, London, U.K. S-2238 was obtained from Kabi-Diagnostics, Stockholm, Sweden. All other reagents were purchased from Sigma.

**Cell culture**

U937 cells were cultured at 37°C in RPMI-1640 medium (Flow Laboratories) containing 10% (v/v) foetal calf serum (FCS, Gibco, Paisley, Scotland, U.K.) and 1.5 mM L-glutamine, in a humidified atmosphere of air/CO\(_2\) (19:1). Cells were divided by a 5–10-fold dilution with culture medium twice a week. In experiments that involved the measurement of the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) or F-actin, U937 cells were harvested and resuspended at a cell density of (4–6) × 10\(^{10}\)/ml in Hepes buffer, pH 7.4, containing 10 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM glucose and 1 mg/ml BSA. Cell viability was greater than 95%, as assessed by Trypan Blue dye exclusion.

**Measurement of [Ca\(^{2+}\)]\(_i\) in U937 cells and human platelets**

Loading of U937 cells with Indo-1/AM has been described elsewhere [16,17]. The preparation of washed human platelets for [Ca\(^{2+}\)]\(_i\) experiments has also been reported in detail elsewhere [16], with the exception that in the present study platelets were loaded with 3 μM Indo-1/AM. U937 cells or platelets (500 μl samples) were incubated at 37°C with stirring, and the fluorescence levels measured using a Shimadzu RF-5000 spectrofluorometer. Fluorescence was monitored at an excitation wavelength of 355 nm, and emission wavelengths of 400 nm and 490 nm. [Ca\(^{2+}\)]\(_i\), was determined by the method of Grynkiewicz et al. [20]. The results were expressed as the maximum change in [Ca\(^{2+}\)]\(_i\), [Δ(Ca\(^{2+}\))]\(_i\), in nM, which was the peak stimulated [Ca\(^{2+}\)]\(_i\)], minus the resting [Ca\(^{2+}\)]\(_i\)].

**Analysis of cellular F-actin content**

The cellular content of polymerized actin (F-actin) was determined by staining of fixed U937 cells with the fluorescent F-actin stain NBD-phallacidin, as previously described [17]. The stained cells were analysed on an Epics Profile fluorescence activated cell sorter (Coulter Electronics). The mean fluorescence intensity per cell was used as a measure of the F-actin content per cell.

**[\(^3\)H]Thymidine incorporation**

The growth of U937 cells was arrested by culture (1 × 10\(^6\)/ml in serum-free RPMI-1640 medium, containing 1 mg/ml BSA and 1.5 mM L-glutamine for 48 h. The cells were then harvested and resuspended in the serum-free RPMI medium. Cell samples (1 ml; 1.5 × 10\(^6\)/ml cells/ml) were plated into 24-well plates (Linbro). Cells were exposed to vehicle, the various forms of thrombin, TRP or 10% FCS for 48 h. After 46 h, the level of thymidine incorporation was assessed by a 2 h pulse of [\(^3\)H]thymidine (1 μCi/ml). The cells were collected, pelleted by centrifugation at 12000 g (MSE Micro-Centaur) for 90 s, washed with 2 × 1 ml of PBS and then lysed with 1 ml of 10% (w/v) trichloroacetic acid for 1 h at 4°C. The trichloroacetic acid-precipitated material was collected following centrifugation at 15000 g for 5 min at 4°C, subjected to 2 × 1 ml washes with ethanol/ether (1:1, v/v) and solubilized with 1 ml of 1 M NaOH. Duplicate 100 μl portions were removed from each sample, neutralized with 1 M HCl and processed for liquid scintillation counting. Results for [\(^3\)H]-thymidine incorporation were expressed as c.p.m./well.

**Preparation of proteolytically inactivated TLCK-thrombin**

Human α-thrombin (3000 units/mg of protein) was inactivated with a 100-fold excess of TLCK, essentially by the method of Sonder and Fenton [21]. The resulting TLCK-thrombin preparation had no residual enzyme activity, as assessed by the hydrolysis of the chromogenic substrate S-2238 [22].

**Statistical analysis**

Unless otherwise indicated, data were analysed using one-way analysis of variance (ANOVA; Statgraphics statistical software package, STSC Inc.). Non-linear curve fitting was performed where necessary for analysis of concentration curves. Data were fitted to a simple one-site model (GraphPad Inplot, GraphPad Software, San Diego, Ca, U.S.A.). Results were considered significantly different when \( P < 0.05.\)
RESULTS

Effects of thrombin, TLCK-thrombin and TRP on Ca\(^{2+}\) mobilization in U937 human monocytic cells and human platelets

One of the earliest events following exposure of cells to thrombin is an increase in [Ca\(^{2+}\)]\(_i\). The results of the present study demonstrate that the hexapeptide TRP (SFLLRN) mimics the ability of thrombin to elevate [Ca\(^{2+}\)]\(_i\) in both U937 human monocytic cells (Figures 1 and 2) and human platelets (Table 1). In platelets, the threshold concentration evoking a significant rise in [Ca\(^{2+}\)]\(_i\) was 50 nM, with the EC\(_{50}\) being 1.18 ± 0.98 μM. This value is similar to the EC\(_{50}\) values previously quoted for TRP-mediated activation of platelets [12]. In contrast, in U937 cells > 5 μM TRP was required to stimulate a significant increase in [Ca\(^{2+}\)]\(_i\), with the EC\(_{50}\) for this response being 86 ± 7 μM.

Structure–function studies in platelets with thrombin receptor peptides of various lengths (6–14 amino acids long) have recently elucidated which amino acid residues are essential for platelet activity [12,14]. These studies have, for example, demonstrated that Phe, Leu and Arg at positions 2, 4 and 5 respectively of the thrombin receptor peptide are essential for activity, with substitution of any one of these resulting in a substantial loss of agonist activity [12,14]. In contrast, substitution of Asn at position 6 results in little loss of agonist activity [14]. Some of these points illustrating the specificity of the TRP were confirmed in U937 cells (Figure 3). Substitution of Asn with Asp at position 6 of the TRP produced a peptide, SFLLRN (TRP-2), which had agonistic effects on intracellular Ca\(^{2+}\) mobilization, albeit with a lower EC\(_{50}\) value (834 ± 416 μM) than that of TRP. However, substitution of Phe with Ala at position 2 of TRP-2 produced a peptide, SALLRD, which failed to stimulate Ca\(^{2+}\) mobilization, even at a concentration of 750 μM (Figure 3).

Both TRP (50 μM–750 μM) and thrombin (2–50 units/ml) evoked comparable increases in [Ca\(^{2+}\)]\(_i\), (Figures 1 and 2), although the kinetics of the TRP-mediated [Ca\(^{2+}\)]\(_i\) response were different from those of the thrombin-mediated [Ca\(^{2+}\)]\(_i\) response (Figures 1 and 2). TRP triggered an immediate rise in [Ca\(^{2+}\)]\(_i\), which peaked within 15 s and returned to resting levels within 60 s, whereas the thrombin-dependent increase in [Ca\(^{2+}\)]\(_i\), was slower and more prolonged, only peaking at 15–25 s and returning to resting levels by 120–180 s.

Desensitization experiments were performed to determine whether TRP acts via the same U937 cell surface receptor as thrombin. In these experiments U937 cells were pretreated with 500 μM TRP or 2 units/ml thrombin for 3 min at 37 °C, by which time [Ca\(^{2+}\)]\(_i\) had returned to resting levels. This was followed by the addition of either 500 μM TRP or 2 units/ml thrombin. Figure 2 demonstrates that thrombin pretreatment resulted in a significant inhibition of Ca\(^{2+}\) mobilization in response to further cell stimulation either with thrombin (75% inhibition compared with thrombin alone) or with TRP (> 60% inhibition compared with TRP alone). TRP pretreatment also resulted in inhibition of the [Ca\(^{2+}\)]\(_i\) response to further cell stimulation with TRP (83% inhibition compared with TRP alone) and to a smaller, though significant, extent with thrombin (> 18% inhibition; P < 0.05 compared with thrombin alone). Thrombin or TRP pretreatment did not, however, effect Ca\(^{2+}\) mobilization in response to another receptor-mediated stimulus, namely platelet-activating factor (results not shown).

With respect to the stimulation of Ca\(^{2+}\) mobilization, these results suggest that TRP acts via the thrombin receptor, since thrombin and TRP desensitize U937 cells to each other (cross-desensitization).

In contrast to native thrombin or TRP, catalytically inactivated TLCK-thrombin (50 and 500 nM) had no effect on [Ca\(^{2+}\)]\(_i\) in U937 cells (Δ[Ca\(^{2+}\)]\(_i\); vehicle alone, 22 ± 8 nM; 50 nM TLCK-thrombin, 18 ± 7 nM; 500 nM TLCK-thrombin, 23 ± 1 nM; means ± S.E.M., n = 6 from two separate experiments). On a unit/ml basis, 50 nM and 500 nM TLCK-thrombin are equivalent to 5.6 units/ml and 56 units/ml of catalytically active thrombin respectively, which are thrombin concentrations which stimulate Ca\(^{2+}\) mobilization in U937 cells (Figures 1 and 2) [16,17]. These TLCK-thrombin concentrations were previously reported to be chemotactic in human blood monocytes [2]. Another inactive thrombin form, PPACK-thrombin, also failed to evoke any increase in [Ca\(^{2+}\)]\(_i\) (results not shown).

Effects of thrombin, TRP and TLCK-thrombin on F-actin formation in U937 cells

The chemotactic response of a mononuclear cell following stimulation requires the continuous rearrangement of its peripheral cytoskeleton. This process is dependent upon the polymerization/depolymerization of cytosolic or globular (G)-actin [23,24]. Earlier workers have shown that thrombin is chemotactic for U937 cells [2], which was indirectly confirmed by work from this laboratory demonstrating that thrombin promotes fila-
mentous (F)-actin formation in U937 cells [17]. Figure 4 demonstrates that, like thrombin, TRP can also stimulate F-actin formation in U937 cells. In response to 500 μM TRP, F-actin formation was rapid and transient, peaking by 10–30 s and returning to basal levels by 5 min. The initial rate of F-actin formation mediated by TRP was, however, higher than that obtained in response to 2 units/ml thrombin, despite the latter stimulating comparable increases in both [Ca2+], (Figures 1 and 2) and F-actin formation (Figure 4a) as 500 μM-TRP. Substantially lower concentrations of TRP were required to evoke F-actin formation compared with Ca2+ mobilization (Figure 1), with the EC50 value for TRP-mediated F-actin formation being 56 ± 27 nM (Figure 4b). The lack of a requirement for a rise in [Ca2+]i for TRP-mediated F-actin formation is in line with our earlier study which reported that thrombin stimulated F-actin formation by a signalling pathway independent of Ca2+ mobilization [17]. In contrast to thrombin or TRP, TLCK-thrombin (500 nM) had no significant effect (P > 0.05) on F-actin formation over a 5 min time course (Figure 4c).

Table 1 Effect of TRP on Ca2+ mobilization in human platelets

<table>
<thead>
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<th>TRP (μM)</th>
<th>Δ[Ca2+]i (nM)</th>
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<td>0</td>
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<tr>
<td>0.05</td>
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<td>624 ± 5*</td>
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<td>5.0</td>
<td>3819 ± 482*</td>
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<tr>
<td>50</td>
<td>3033 ± 313*</td>
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<tr>
<td>Thrombin (2 units/ml)</td>
<td>2818 ± 291*</td>
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Figure 2 Effect of TRP or thrombin pretreatment on Ca2+ mobilization in response to further TRP or thrombin stimulation

Cells were initially challenged with either 500 μM TRP (a and b) or 2 units/ml thrombin (c and d) for 3 min and then re-challenged with a further dose of 500 μM TRP (a and d) or 2 units/ml thrombin (b and e). The values beside each trace denote the maximum change in [Ca2+]i (nM) following agonist addition. All other conditions are as described in the legend in Figure 1. *P < 0.05 compared with the peak [Ca2+]i, attained with the first agonist (Student’s t-test).

Figure 3 Effect of the hexapeptides SFLLRD and SALLRD on Ca2+ mobilization in U937 cells

U937 cells were stimulated with either the hexapeptide SFLLRD (■) or the hexapeptide SALLRD (∆) and the maximum change in [Ca2+]i was determined. All other conditions were as described in the legend to Figure 1. *P < 0.05 compared with vehicle.
U937 human monocyte activation by a thrombin-receptor-derived hexapeptide

Effects of the thrombin derivatives and TRP on [3H]thymidine incorporation in U937 cells

Experiments were performed to address the possibility of a mitogenic response by U937 cells to thrombin, TRP or the inactive thrombin derivatives. U937 cells were growth-arrested for 48 h in serum-free medium and exposed to the various stimuli for 48 h, and [3H]thymidine incorporation was determined in the penultimate 2 h. As shown in Figure 5, thrombin (10–50 units/ml) caused a concentration-dependent 2–8-fold increase in [3H]-

Figure 4 Effects of thrombin, TRP and TLCK-thrombin on F-actin formation in U937 cells

The F-actin content of cells stimulated with vehicle or agonist was determined by staining with the fluorescent F-actin dye NBD-phallacidin, followed by flow cytometric analysis. (a) Time course experiments with vehicle (○), 500 µM TRP (●) and 2 units/ml thrombin (△). (b) Concentration–response effects of TRP. (c) Time course effects of vehicle (○) or 500 nM TLCK-thrombin (△). Results are the means ± S.E.M. from 2–3 separate experiments. *P < 0.05 compared with vehicle.

Figure 5 Effect of thrombin, TRP and the thrombin B-chain-derived Peptides 1 and 2 on [3H]thymidine incorporation in U937 cells

Growth-arrested U937 cells were treated with (a) thrombin, (b) TRP or (c) Peptide 1 (●) or Peptide 2 (△) for 48 h, and the level of [3H]thymidine incorporation was determined in the penultimate 2 h of stimulation. The results from the thrombin, TRP and thrombin B-chain peptide experiments are the means ± S.E.M. and were obtained respectively from three (n = 8–9 determinations), two (n = 6) and four (n = 11–12) experiments. *P < 0.05 compared with vehicle.
thymidine incorporation. However, the threshold concentration of thrombin (≈ 10 units/ml) causing significant (P < 0.05) mitogenic effects was greater than the threshold thrombin concentration required to stimulate [Ca]\textsuperscript{2+}, or F-actin formation (Figures 1, 2 and 4) [16,17]. Similarly, TRP only had a significant (P < 0.05) mitogenic effect (1.4-fold above resting levels) at ≧ 500 μM TRP, which again was considerably higher than the threshold TRP concentration required to stimulate Ca\textsuperscript{2+} mobilization or F-actin formation (Figures 1, 2 and 4).

Two synthetic 14-amino-acid peptides (Peptides 1 and 2) based on part of the B-chain of thrombin [3,4] were examined for their potential mitogenic effects in U937 cells. At concentrations of Peptide 1 (10 nM–10 μM) reported to be mitogenic in murine macrophage cell lines [3,4], there was no significant (P < 0.05 compared with vehicle) increase in [3H]thymidine incorporation in U937 cells (Figure 5). However, under the same experimental conditions, these cells responded mitogenically to FCS or thrombin ([3H]thymidine incorporation (c.p.m./well): vehicle, 1393 ± 338; + 10% FCS, 22914 ± 6122; + 50 units/ml thrombin, 10969 ± 2812). As the mitogenic effects of Peptide 1 had been shown previously to occur in macrophage-like cells, the possibility was considered that U937 cells might also respond only to Peptide 1 in their macrophage-like phenotype. However, in experiments employing U937 cells differentiated to a macrophage-like phenotype [16,17], Peptide 1 still had no mitogenic effect (results not shown). None of Peptide 2 (10 nM–10 μM) (Figure 5), TLCK-thrombin or PPACK-thrombin (results not shown) had any mitogenic effect in U937 cells.

**DISCUSSION**

The recent cloning of the thrombin receptor has revealed that thrombin-stimulated responses in platelets, endothelial cells and fibroblasts are mediated by a mechanism dependent upon the proteolytic cleavage of its receptor [6–8]. The observation that human blood monocytes and various murine macrophage cell lines could be activated by catalytically inactivated forms of thrombin or peptides corresponding to parts of the thrombin B-chain [2–4] suggested that monocyte cells might possess a second class of thrombin receptor that is activated by a non-proteolytic, hormone-like mechanism. However, in the present study using the thrombin-responsive U937 human monocyte cell line [2,16,17], evidence was obtained solely for a proteolytic mechanism for thrombin cell activation. The inactive thrombin derivatives TLCK-thrombin and PPACK-thrombin had no effect on [Ca\textsuperscript{2+}], F-actin formation or cell proliferation in U937 cells, which was clearly different to the responses mediated by native thrombin. Further, a synthetic 14-amino-acid peptide (Peptide 1) corresponding to a part of the B-chain of thrombin, which has been reported to be mitogenic in murine macrophage cell lines [3,4], also had no mitogenic effect in U937 cells. Species differences might be invoked as a reason for the difference in effects of the various catalytically inactive forms of thrombin reported in this study using the U937 human monocyte-macrophage cell line (no agonistic effects) and in the earlier studies of Bar-Shavit et al. [3,4] in murine macrophage cell lines (agonistic effects).

In contrast to the inactive forms of thrombin, the N-terminal thrombin-receptor-derived hexapeptide (TRP; SFLLRN) [12,14] stimulated the same U937 cell responses as did native catalytically active thrombin. The U937 human monocyte/macrophage cell line therefore represents another thrombin-responsive cell system which requires proteolytic activation of the thrombin receptor, and which can be activated by thrombin-receptor-derived peptides. There were, however, subtle differences between the effects of TRP and thrombin. For example, the initial rates of Ca\textsuperscript{2+} mobilization and F-actin formation were higher in response to TRP relative to thrombin. The faster action of TRP could reflect the fact that this peptide can activate the thrombin receptor directly, whereas the delayed action of thrombin reflects the time required for thrombin to bind to the receptor and generate sufficient tethered receptor–ligand for receptor activation.

Another apparent difference between TRP and thrombin was the observation that TRP pretreatment only partially desensitized U937 cells to further stimulation with thrombin, whereas thrombin pretreatment caused considerably greater desensitization to further thrombin stimulation. In this context, a recent study in HEL cells showed that thrombin receptor desensitization occurs by two mechanisms [13]. Desensitization required (i) proteolysis at one or more sites of the thrombin receptor, and (ii) protein phosphorylation, possibly of the receptor itself. While thrombin triggered both mechanisms in HEL cells, a 14-amino-acid thrombin-receptor-derived peptide was only able to trigger the protein-phosphorylation-dependent pathway of desensitization [13]. If a similar situation to that seen in HEL cells [13] also occurred in U937 cells, the inability of the TRP to trigger the proteolytic pathway of desensitization could explain its weaker desensitization effect.

Whether all the cellular actions of thrombin require hydrolysis of its receptor remains to be established. In macrophage cells of murine origin [3,4] the possibility was considered of other thrombin receptor subtypes which do not require the serine proteinase activity of thrombin for cell activation. This possibility has, however, been ruled out in the present study using U937 human monocyte/macrophages, and to date (with one exception) all of the cellular actions of thrombin have been shown to be mimicked by synthetic 5–14-amino-acid peptides corresponding to a part of the newly created N-terminal domain of the cleaved thrombin receptor [6–14]. The one exception was a study by Vouret-Craviari et al. [9], who demonstrated that thrombin-receptor-derived peptides failed to mimic the mitogenic effect of thrombin in CCL39 hamster fibroblasts. These workers suggested that the mitogenic action of thrombin in CCL39 cells might be mediated by another class of thrombin receptor, one that is not activated by thrombin-receptor-derived peptides. However, Huang et al. [11] have more recently reported mitogenic effects of thrombin-receptor-derived peptides in CCL39 cells. The present study has also shown that TRP mimics the mitogenic effect of thrombin in U937 cells, though for both thrombin and TRP considerably higher concentrations were required to stimulate a threshold mitogenic response relative to other U937 cell responses.

In an earlier study from this laboratory, we reported differences in the behaviour of thrombin with U937 cells relative to human platelets [16]. In U937 cells, though not in platelets, thrombin failed to stimulate thromboxane B\textsubscript{2} synthesis or to inhibit adenylate cyclase. Additionally, concentration–response curves for Ca\textsuperscript{2+} mobilization in the two cell systems revealed that the thrombin concentration required to evoke a threshold response in U937 cells was ≈ 20-fold higher than in platelets. Interestingly, in the present study, differences in the concentration–response curves for TRP-stimulated Ca\textsuperscript{2+} mobilization in platelets and U937 cells were also evident, e.g. the threshold TRP concentration was ≧ 100-fold greater in U937 cells compared with platelets. While the differences between U937 cells and platelets reported here and elsewhere [16] with respect to the effects of thrombin and TRP do not provide evidence that thrombin receptor subtypes exist, it is tempting to speculate that U937 cells possess a subtype of the platelet thrombin receptor which is also a substrate for thrombin serine proteinase activity, but at which
the newly revealed N-terminus ligand has lower affinity for the receptor. This possibility, however, requires further examination.

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