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Characterization of the Binding of Bovine Thrombin to Isolated Rat Hepatocytes

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Key words

Thrombin – Receptor – Thrombin-binding site – High-affinity binding – Rat hepatocyte

Summary

Isolated rat hepatocytes possess per cell 4,500 high-affinity binding sites for thrombin with a K_d of 30–40 pM, and 2.8×10^5 low-affinity sites with a K_d of 30 nM. These binding sites are highly specific for thrombin. Half-maximal binding of ^{125}I-labelled thrombin is achieved after 3 min at 37° C and 7 min at 4° C. The reversibly bound fraction of the ligand dissociates according to a biexponential time course with the rate constants 1–2 $\times 10^{-2}$ s^{-1} and 3–4 $\times 10^{-4}$ s^{-1}. Part of the tracer remains cell-associated even after prolonged incubation, but all cell-associated radioactivity migrates as intact thrombin upon sodium dodecyl sulphate polyacrylamide gel electrophoresis. The bound thrombin is minimally endocytosed as judged by the resistance to pH 3-treatment. Cell-associated radioactivity dissociated from the cells binds just as well in a receptor assay as tracer incubated in a conditioned medium under the same conditions, indicating the absence of a quantitatively important receptor-mediated degradation of the ligand.

Introduction

The coagulation enzyme thrombin is mainly cleared from the circulation by a receptor-mediated uptake in hepatocytes after it has formed a complex with a protease inhibitor (1). Although thrombin will form a complex mainly with antithrombin III (2–4), some will be attached to other protease inhibitors like α_2-macroglobulin (3–5) and α_1-antitrypsin (α_1-proteinase inhibitor) (3, 5), and cleared by specific receptors for these protease inhibitor-protease complexes (6–8). However, a number of cell types possess receptors for uncomplexed thrombin. Thus, receptor binding of thrombin to platelets leads to degranulation and aggregation of these cells (9–11), receptor-bound thrombin stimulates the growth of fibroblasts (12–14), and also endothelial cells possess receptors for thrombin (15).

The aim of the present study was to investigate whether collagenase-isolated rat hepatocytes can bind thrombin independently of the formation of complexes with protease inhibitors. In this report, the binding kinetics of thrombin to high-affinity receptors in rat hepatocytes is characterized. While this study was in progress, the presence of a displaceable association of ^{125}I-labelled thrombin to hepatocytes was demonstrated, although not characterized in detail (16). A preliminary account of some of the results has been published in abstract form elsewhere (17).

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Materials and Methods

Thrombin and Thrombin Derivatives

Bovine α-thrombin was obtained as previously described (18) and repurified on a column of sulphopropyl-Sephadex C-50 (Pharmacia) (19). This material gave a single band when subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis.

The thrombin was routinely radiolabelled with ^{125}I by incubating 2.5 µl of thrombin (26.3 µM in 0.6 M NaCl buffered to pH 6.0 with 0.1 M Na-phosphate) with 7.4 MBq (in 2–4 µl) ^{125}I (Amersham International) on ice. Oxidation of the iodide was achieved by adding 5 µl chloramine-T (0.3 mM) followed by another 5 µl after 1 min. One minute later, the reaction was terminated by the addition of 10 µl of $Na_2S_2O_5$ (0.44 mM). Alternatively, thrombin was iodinated with solid-phase lactoperoxidase-glucose oxidase (Enzymobead, Bio-Rad) according to the manufacturer.

The iodinated thrombin was purified by chromatography on a 1 × 18 cm column of Sepharose CL-6B (Pharmacia) using a Krebs-Ringer-Hepes buffer fortified with 10 g/l of bovine serum albumin (fraction V, Sigma) and 0.6 M NaCl, pH 6.0. Additionally, the tracer was passed over a Sephadex G-50 Fine (Pharmacia) column (bed volume 2 ml) prior to each experiment. Thus, a tracer with a specific activity of 5–6 $\times 10^{16}$ Bq/mol (degree of iodination about 0.7) was obtained.

Non-saturable binding was assessed by the addition of 50 NIH Units/ml of Thrombin Reagent Leo, a commercial bovine thrombin preparation (a generous gift from the Hormone Department, Leo Pharmaceutical Products, Ballerup, Denmark).

Prethrombin 1 (Ser^{157}-Ser^{582} of the prothrombin sequence) was obtained by digestion of prothrombin with a catalytic amount of thrombin followed by purification on a DEAE-Sephadex A-50 (Pharmacia) column as described (20). Prethrombin 1 (5 mg in 1 ml of 50 mM NH_4HCO_3) was activated to meizothrombin 1 by 5 µg ecarin at room temperature. After 1 hour, the reaction was complete as judged by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and the activation was stopped by freezing. The purified prothrombin activator (ecarin) from the venom of *Echis carinatus* was a gift from Dr. F. Kornalik.

Cell Preparations

Hepatocytes were isolated by in vitro perfusion with collagenase (type I, Worthington) of the liver from male rats, fed ad libitum and weighing 190–230 g, by a modification (21) of the method of Berry and Friend (22). The parenchymal liver cells were separated from non-parenchymal and damaged cells by centrifugation on a preformed linear gradient of Percoll (Pharmacia) (23, 24). Except for the perfusion, all procedures were performed at 4° C.

Plasma membranes were prepared from collagenase-isolated rat adipocytes (25) by a previously described method using self-generating gradients of Percoll (26).

Incubations

The cells and membranes were incubated in a Krebs-Ringer salt solution buffered to pH 7.4 with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes) and supplemented with 10 g/l of dialyzed bovine serum albumin (25). Other conditions were as described in the legends to figures and tables.

All cell incubations were stopped by centrifugation of 0.2 ml aliquots of the cell suspension through a layer of dibutyl phthalate/dinonyl phthalate (2:1, v/v) (BDH) in a Beckman Microfuge. The cell pellet was isolated by cutting the tube through the oil layer (27).

Table 1 Binding of ^{125}I-labelled thrombin iodinated by the lactoperoxidase or the chloramine-T catalyzed methods

	Chloramine-T B/F	Lactoperoxidase
Total binding	0.093 ± 0.002	0.113 ± 0.013
Non-saturable binding	0.010 ± 0.001	0.061 ± 0.004
Saturable binding	0.083	0.052

Isolated rat hepatocytes (8 × 10^5/ml) were incubated with 20 pM of ^{125}I-labelled thrombin iodinated by either the lactoperoxidase or by the chloramine-T methods. Total binding represents the radioactivity found in cells incubated with tracer alone, and the non-saturable binding in cells incubated with a surplus of unlabelled thrombin in addition to the same concentration of tracer. The difference is denoted saturable binding. The data are expressed as Bound in fraction of Free (i. e. unbound) radioactivity. Since there was a great batch to batch variation for the lactoperoxidase iodinated thrombin preparations, the results of one set of parallel iodinations are used as an illustration. Details are given in Materials and Methods. Mean ± S. D. of four replicates

The endocytosed fraction of the cell-associated radioactivity was determined as the radioactivity remaining cell-associated after exposure of the cells to icecold barbitone buffer at pH 3 for 3 min (28) prior to centrifugation through oil.

Degradation of the ligand was assessed by its solubility in 0.75 M trichloroacetic acid or by the loss of ability to bind to thrombin receptors on rat adipocyte plasma membranes. Membrane incubations were stopped by filtration through hydrophilic polyvinylidene fluoride membrane filters (type GVWP, Millipore) followed by washing twice with 2.5 ml icecold buffer containing 10 g/l bovine serum albumin.

Other Methods

Radioactivity was quantitated in a Selectronic γ-spectrometer with an efficiency of 0.49, and a background of 6 cpm.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (reagents from Fluka) was carried out in the Laemli discontinuous buffer system without reduction (29). Slab gels were stained with Coomassie brilliant blue R250 (Merck), destained and dried, and the radioactive bands were visualized by autoradiography using Hyperfilm-MP (Amersham) and enhancing screens. Alternatively, rod gels were cut into 1.5 mm slices and quantified for ^{125}I activity.

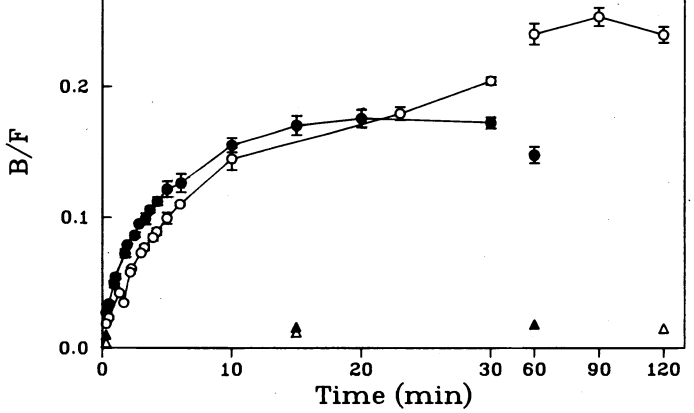


Fig. 1 Association of ^{125}I-labelled thrombin. Isolated rat hepatocytes (1 × 10^6 cells/ml) were incubated with 25 pM ^{125}I-labelled thrombin at either 37° C (closed symbols) or 4° C (open symbols) under slow magnetic stirring. At the indicated times, 4 aliquots of 200 µl were centrifuged through oil as described in Materials and Methods. The non-saturable binding was assessed by the addition of a surplus of unlabelled thrombin (triangles). Bars represent S. D. when exceeding the size of the symbol

Other Materials

Hirudin was purified as described (30) and was a gift from Dr. D. Bagdy. Human α_2-macroglobulin (a gift from Dr. Lars Sottrup-Jensen) was prepared from outdated, pooled citrate plasma using Zn^{2+}-chelate affinity chromatography and complexed with trypsin as previously described (31, 32).

The fibronectin tetrapeptide (Arg-Gly-Asp-Ser) was purchased from Cambridge Research Biochemicals, heparin from Leo Pharmaceuticals, porcine pancreatic elastase and bovine α_1-antitrypsin from Boehringer Mannheim, and urokinase (Ukidan) from Serono. Other biochemicals were from Sigma. All inorganics were of analytical grade obtained from Merck.

Results

Evaluation of the ^{125}I-Labelled Thrombin Preparation

When thrombin was iodinated by the lactoperoxidase-glucose oxidase method, a large proportion of the radioactivity eluted in the void volume of the Sepharose CL-6B column. This was not seen, when chloramine-T was used as the oxidizing agent in the iodination procedure (data not shown).

When hepatocytes were incubated with radioactivity eluting from the column at the position of thrombin, a higher fraction of the lactoperoxidase-catalyzed tracer bound to the cells than seen after a parallel chloramine-T iodination (Table 1). However, the increase in the non-saturable binding (i. e. the binding of ^{125}I-labelled thrombin in the presence of a surplus of unlabelled thrombin) exceeded the increase in the total binding. Consequently, the displaceable or specific binding was lower with the lactoperoxidase than with the chloramine-T-oxidized tracer (Table 1). For these reasons, all subsequent experiments were performed with tracer iodinated with the chloramine-T method. Attempts to increase the degree of iodination above unity decreased the binding affinity of the tracer (data not shown). Thrombin from three different batches bound with the same kinetics (data not shown).

Association

The binding of 25 pM ^{125}I-labelled thrombin to isolated rat hepatocytes reached a plateau after 20 min at 37° C and after about 60 min at 4° C (Fig. 1). The half-times were 3 min (range in 4 experiments 3–4 min) at 37° C and 7 min (range in 4 experiments 6–8 min) at 4° C. After prolonged incubation at 37° C, the cell-associated radioactivity decreased (not shown).

Dissociation

The addition of a surplus of unlabelled thrombin to cells incubated with ^{125}I-labelled thrombin for 30 min at 37° C or 60 min at 4° C showed that the binding process was partially reversible. Half of the radioactivity was dissociated after 3 min (range in 3 experiments 2–3.5 min) at 37° C, and after 16 min (range 9–20 min) at 4° C. However, the dissociation curve reached a plateau leaving 0.17 (range 0.11–0.25) of the cell-associated radioactivity irreversibly attached to the cells at 37° C, and 0.27 (0.25–0.28) at 4° C. Even when subtracting this irreversible component, the remaining radioactivity did not follow a monoexponential time course of dissociation (data not shown).

In order to evaluate the time course of dissociation quantitatively, isolated hepatocytes were incubated with ^{125}I-labelled thrombin as above. The cells were concentrated by centrifugation and resuspended in buffer at the same temperature containing no or a surplus of unlabelled thrombin. At 4° C, the presence of a

surplus of thrombin in the dissociation medium had no effect on the time course of dissociation of the fraction of label dissociating (Fig. 2). However, in the absence of thrombin only 0.59 dissociated, whereas 0.92 dissociated in the presence of unlabelled thrombin. The data could adequately be described by a biexponential time course (Fig. 2). The compiled data from four experiments are presented in Table 2. At 37° C, the presence of unlabelled thrombin had an accelerating effect on the slow component of dissociation and also reduced the fraction of irreversibly bound tracer (Table 2). Again the data could be described by a biexponential time course (Fig. 3 and Table 2).

Concentration Dependence of the Thrombin Binding

The concentration dependence of the binding of thrombin to isolated hepatocytes revealed a receptor heterogeneity. Half-maximal binding was attained at 23 ± 3 pM at 4° C and at 54 ± 27 pM thrombin at 37° C (mean ± S. E., n = 4) (Fig. 4). When the data were plotted according to Scatchard (Fig. 4, inset), the resulting curve could be resolved into at least two components. A high affinity component with an apparent K_d of 31 ± 4 pM at 4° C and 40 ± 23 pM at 37° C constituted 0.02 of the total number of sites. A low affinity component was found to possess an apparent K_d of 34 ± 15 nM and 26 ± 21 nM at 4° C and 37° C, respectively (mean ± S. E. of 4 independent experiments). There was a total of 2.8 ± 1.4 × 10^5 binding sites per cell.

pH Dependency of Thrombin Binding

In the buffer used, the binding of thrombin was independent of pH in the range 6–8. At pH values higher than 8, both the total binding and the non-saturable binding increased (data not shown).

Endocytosis of the Bound Thrombin

At 37° C, about 0.3 of the specifically bound ^{125}I-labelled thrombin remained cell-associated after a brief incubation in an ice-cold stopping solution with pH 3. The pH 3-resistance (Fig. 5, closed squares) developed more slowly than the association of the tracer to the cells (Fig. 5, closed circles). By contrast, at 4° C the pH 3-resistant radioactivity was of the same magnitude as the non-saturable binding at all times investigated (Fig. 5).

Degradation of Thrombin

When isolated hepatocytes were incubated with ^{125}I-labelled thrombin at 37° C, the trichloroacetic acid solubility of the radioactivity increased linearly with time without any measurable lag. The rate of appearance of the trichloroacetic acid soluble products was the same as that seen when the tracer was incubated at 37° C in the absence of cells (data not shown). Thus, the role of the hepatic thrombin receptor in the degradation of thrombin appears to be minimal.

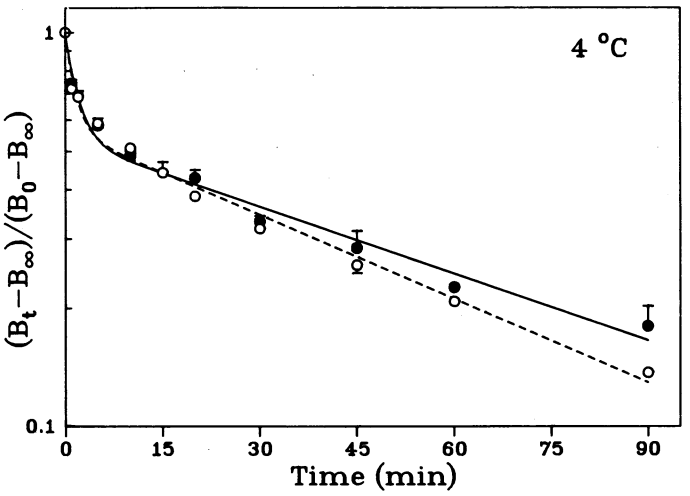


Fig. 2 Dissociation of ^{125}I-labelled thrombin at 4° C. Isolated rat hepatocytes (8 × 10^5 cells/ml) were incubated with 15 pM ^{125}I-labelled thrombin for 60 min at 4° C under slow magnetic stirring. The cell-associated radioactivity was determined (B_0). The remaining cell suspension was concentrated to about 1 ml after gentle centrifugation and further concentrated by a brief spin through oil in a Beckman Microfuge. The cell pellet was immediately cut and resuspended in buffer containing no (●), or a surplus of unlabelled thrombin (○). At the indicated times, 3 aliquots of 200 µl were centrifuged through oil, and the cell-associated radioactivity determined (B_t). The non-dissociable radioactivity (B_∞) was subtracted from all values prior to calculation. A two compartment model was fitted to the experimental data using an iterative program. Compiled data from four independent experiments are presented in Table 2. Bars represent S. D. when exceeding the size of the symbol

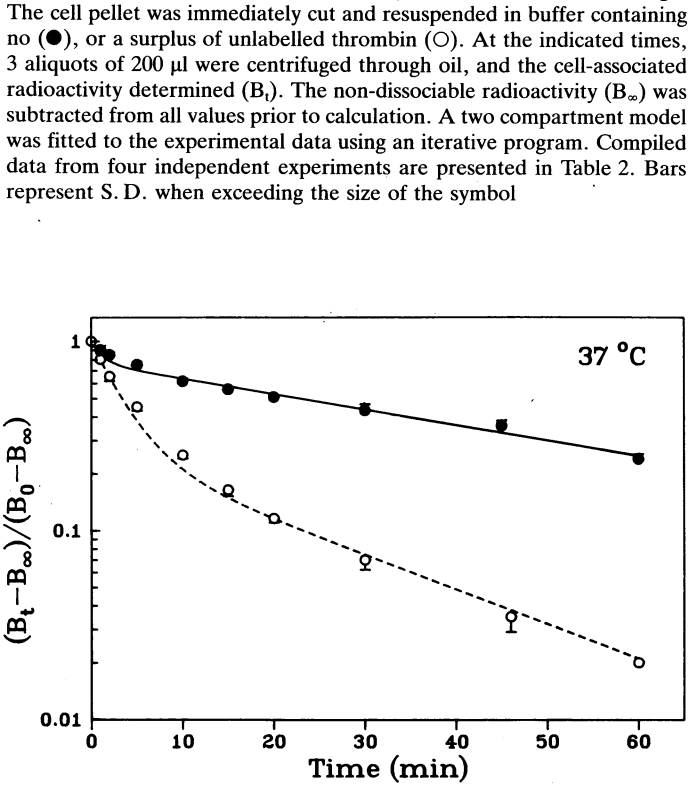


Fig. 3 Dissociation of ^{125}I-labelled thrombin at 37° C. The experimental details and symbols are as given in the legend to Fig. 2 except that the cells were incubated at 37° C for 30 min prior to initiation of the dissociation

Table 2 Dissociation of ^{125}I-labelled thrombin from isolated rat hepatocytes

	Unlabelled thrombin	Fraction	k′ (s^{-1}) × 10^2	Fraction	k″ (s^{-1}) × 10^4	Irreversibly bound fraction
4° C	0	0.45 ± 0.05	2.2 ± 0.5	0.55 ± 0.05	3.7 ± 0.9	0.41 ± 0.02
4° C	+	0.54 ± 0.01	3.5 ± 0.8	0.46 ± 0.01	3.3 ± 0.3	0.08 ± 0.01
37° C	0	0.38 ± 0.04	1.4 ± 0.2	0.62 ± 0.04	3.2 ± 0.4	0.15 ± 0.01
37° C	+	0.66 ± 0.03	1.2 ± 0.3	0.34 ± 0.03	9.2 ± 0.9	0.04

Isolated rat hepatocytes were incubated as described in the legends to Figs. 2 and 3. The irreversibly bound fraction of the tracer was subtracted, and the remaining radioactivity plotted as a function of time as in Figs. 2 and 3. A biexponential time course was fitted to the experimental data by an iterative program. Mean ± S. E. of four independent experiments.

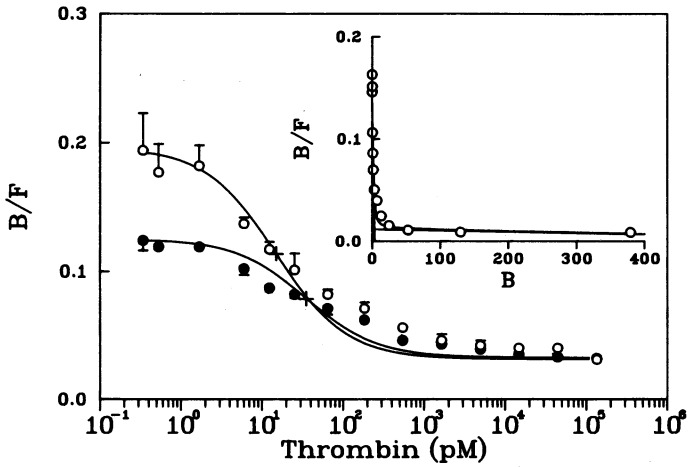


Fig. 4 Concentration dependence of the binding of thrombin to isolated rat hepatocytes. Isolated rat hepatocytes (8×10^5 cells/ml) were incubated with ^{125}I-labelled thrombin at a constant specific activity in the range 0.4–7 pM or with 7 pM ^{125}I-labelled thrombin plus unlabelled highly purified thrombin to give the indicated concentration. After 30 min at 37° C (●) or 120 min at 4° C (○) in a shaking waterbath, the incubation was stopped by centrifugation of the cells through oil as described in Materials and Methods. The curves describe the best computerized fit of a one compartment model to the experimental data. Half maximal binding is marked by +. The data deviate systematically from the curve, indicating a receptor heterogeneity. The inset shows the 4° C data plotted according to Scatchard after subtraction of the non-saturable binding (i.e. the binding in the presence of 140 nM thrombin). The abscissa is expressed as bound thrombin in pmol/l cell suspension. The lines represent the best computerized fit of a two compartment model to the data. The compiled data from four independent experiments are given in the text. Bars represent S.D. (n = 4) when exceeding the size of the symbol

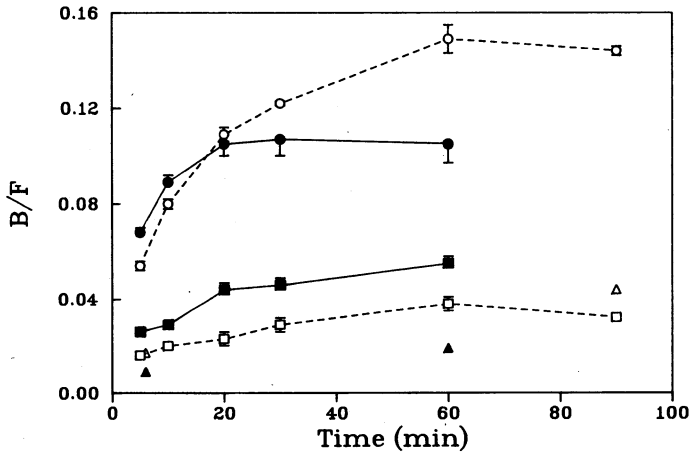


Fig. 5 Resistance of the bound ^{125}I-labelled thrombin to exposure to pH 3. Isolated rat hepatocytes (1×10^6 cells/ml) were incubated with 25 pM ^{125}I-labelled thrombin at either 37° C (closed symbols) or 4° C (open symbols) under slow magnetic stirring. At the indicated times, 3 aliquots of 200 µl were centrifuged through oil (circles and triangles) in a 550 µl microfuge tube, or mixed with 1 ml of icecold barbitone buffer, pH 3 (28), for 3 min in a 1.5 ml microfuge tube, followed by the addition of oil, and centrifugation (squares). The non-saturable binding was assessed by the addition of a surplus of unlabelled thrombin (triangles). This non-saturable binding was after treatment at pH 3 (not shown) of a size similar to that shown for samples stopped by oil-centrifugation only. Bars represent S.D. when exceeding the size of the symbol

This was confirmed by the receptor binding ability of dissociated thrombin. Hepatocytes were incubated with ^{125}I-labelled thrombin, isolated from the medium, and resuspended in fresh buffer without thrombin. After a period of dissociation, the medium was isolated and incubated at 4° C with adipocyte plasma membranes. The majority of the dissociated radioactivity could be rebound, and this binding was displaceable by unlabelled thrombin to the same extent as the fresh tracer (Table 3). The ability to rebind to the plasma membranes was higher after dissociation at 4° C than at 37° C. However, the rate of degradation was similar in a conditioned medium (i.e. the tracer incubated in a cell-free supernatant previously exposed to cells for the same duration) (Table 3). Since the radioactivity released immediately after initiation of the dissociation could represent unprocessed thrombin, the dissociation period was divided into two at 37° C. However, the radioactivity released later, and thus representing some of the slowly dissociating radioactivity (cf. Fig. 3), bound with the same affinity as did the radioactivity released during the initial 10 min of the dissociation (Table 3). This indicates the absence of an extensive receptor-mediated degradation.

Molecular Integrity of the Thrombin Tracer in the Incubates

In order to exclude that the observed displaceable cell-association of ^{125}I-labelled thrombin occurred to receptors for protease inhibitor-protease complexes, the nature of the radioactivity was investigated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Fig. 6). Both dissociated tracer previously bound to hepatocytes (Fig. 6A, lane A) and tracer incubated in a conditioned medium (Fig. 6A, lane C) or just in buffer (Fig. 6A, lane B) moved as a single component with the same mobility as freshly purified ^{125}I-labelled thrombin (Fig. 6A, lane D). Thus, in a situation where more than 0.2 of the added tracer is taken up by the cells, it is unlikely that this should occur as other species than the only detectable, thrombin. This was shown directly by solubilizing cell pellets in 20 g/l of sodium dodecyl sulphate after incubation to a steady state with ^{125}I-labelled thrombin and subjecting this extract to sodium dodecyl sulphate polyacrylamide gel electrophoresis. Again, the migration of the cell-associated radioactivity did not deviate from that of ^{125}I-labelled thrombin (Fig. 6B).

Table 3 Degradation of ^{125}I-labelled thrombin by isolated rat hepatocytes

	n	Receptor binding (relative to control)
Fresh tracer, control	7	1
4° C, 0–45 min	7	0.90 ± 0.03
37° C, 0–10 min	7	0.75 ± 0.06
37° C, 10–30 min	4	0.72 ± 0.07
37° C, conditioned medium	5	0.63 ± 0.08

Isolated rat hepatocytes (1.6×10^6 cells/ml) were incubated for 30 min at 37° C or for 60 min at 4° C with 0.1 nM ^{125}I-labelled thrombin. The cells were isolated by centrifugation through oil as described in Materials and Methods and resuspended in buffer at the same temperature without added thrombin. After the indicated time of dissociation, the cell-free supernatant was isolated and incubated with rat adipocyte plasma membranes (3 µg protein/ml) for 2 h at 4° C. For comparison, a conditioned medium (i.e. incubation and handling of the cells similar to the 37° C experiments) was prepared, but the ^{125}I-labelled thrombin was only incubated with this cell-free conditioned medium for 10 min at 37° C in order to assess the cell-independent degradation of thrombin. Mean ± S.E. of n independent experiments performed in triplicates.

Specificity of the Thrombin Receptor Interaction

Prothrombin and prethrombin 1 (i.e. residues Ser^{157}-Ser^{582} of the prothrombin sequence) did not in a concentration of 150 nM interfere with the binding of ^{125}I-labelled thrombin (Table 4). However, activation of prethrombin 1 to meizothrombin 1 (i.e. Ser^{157}-Arg^{323} disulfide bound to Ile^{324}-Ser^{582}) by ecarin introduced the ability to interact with the thrombin binding (Table 4). This was also shown by ^{125}I-labelling of prethrombin 1 and meizothrombin 1. Only the latter bound in a displaceable manner (data not shown).

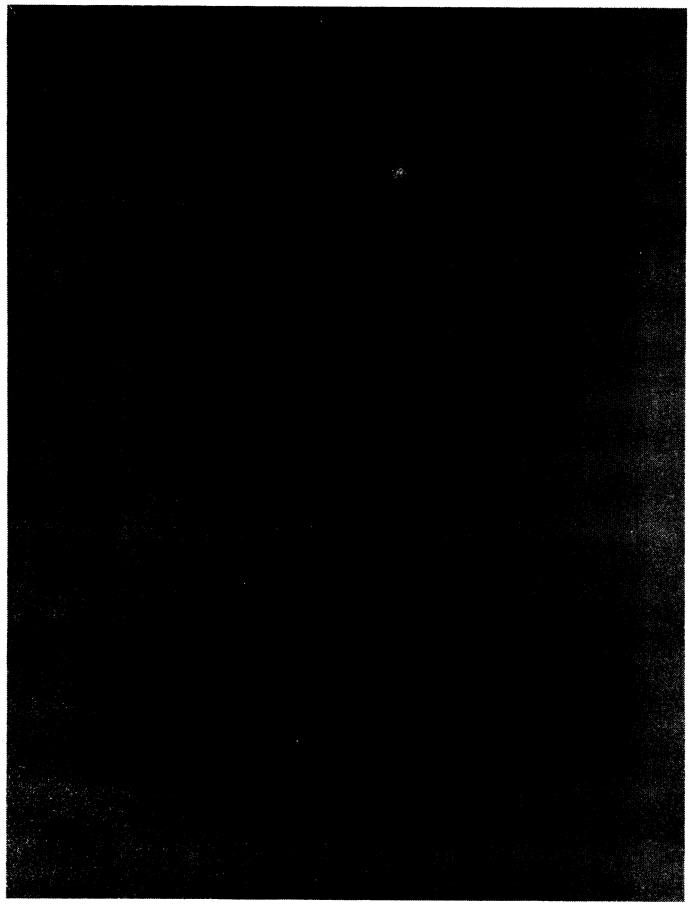
Table 4 Specificity of the thrombin receptor interaction

Agent	Concentration	n	Relative receptor binding
None, control			1
Thrombin	150 nM	8	0.27 ± 0.04
Prothrombin	150 nM	5	0.95 ± 0.03
Prethrombin 1	150 nM	4	0.85 ± 0.03
Meizothrombin 1	150 nM	4	0.35 ± 0.03
Arg-Gly-Asp-Ser	100 μM	2	0.91–1.08
Urokinase	25 μg/ml	2	0.68–0.85
Elastase	190 nM	2	0.91–1.03
α_1-Antitrypsin-elastase	1 U/ml	2	0.84–0.93
α_2-Macroglobulin-trypsin	110 nM	2	0.82–1.03
Benzamidine	10 mM	2	0.75–0.90
Phenylmethanesulphonyl fluoride	1 mM	2	0.74–1.02
Hirudin	10 U/ml	2	0.09–0.11
Heparin	50 U/ml	2	0.12–0.24
EDTA	5 mM	2	0.90–1.22

Isolated rat hepatocytes (1.6×10^6 cells/ml) were incubated for 90 min at 4° C with 20 pM ^{125}I-labelled thrombin alone (i.e. control) or plus the indicated agents. The cells were isolated by centrifugation through oil as described in Materials and Methods. The α_1-antitrypsin-elastase complex was made with an excess of elastase. Mean ± S.E. of n independent experiments or the range of two experiments each performed in four replicates.

The sequence Arg-Gly-Asp is found in the thrombin molecule corresponding to residues 520–522 of the prothrombin sequence. The Arg-Gly-Asp sequence has been shown to represent the receptor recognition site of fibronectin, vitronectin, type I collagen, fibrinogen, von Willebrand factor, and the protein C3 from the complement system (33–38). Isolated rat hepatocytes possess receptors for fibronectin (39). However, in contrast to these adhesive molecules, the tetrapeptide Arg-Gly-Asp-Ser could not prevent the binding of ^{125}I-labelled thrombin in the present system (Table 4).

Urokinase and elastase did not interfere with the binding. α_1-Antitrypsin-elastase complex (in a surplus of elastase) and α_2-macroglobulin-trypsin complex were also without any effect. The enzyme inhibitors benzamidine and phenyl-methanesulphonyl fluoride did not change the binding of ^{125}I-labelled thrombin (Table 4), indicating that this occurred independently of a preserved catalytic activity. However, the thrombin-specific



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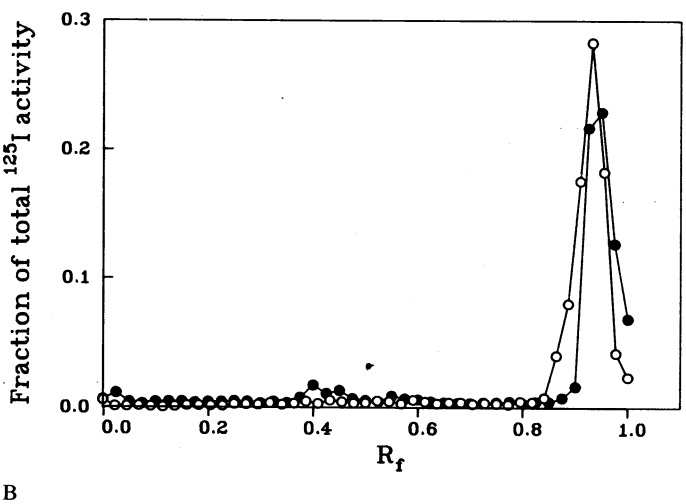

B

Fig. 6 Electrophoretic mobility of the tracer after incubation with cells and media. *A.* Hepatocytes (1×10^6 cells/ml) were incubated in the presence of 100 pM ^{125}I-labelled thrombin for 30 min at 37° C. The cells were isolated and resuspended in buffer containing 1 g/l of bovine serum albumin. Lane A shows the dissociated radioactivity isolated as described in the legend to Table 3. ^{125}I-labelled thrombin was incubated for 30 min at 37° C in buffer containing 10 g/l of albumin (lane B), or in a cell-free supernatant of a conditioned medium from cells incubated in buffer containing 10 g/l of albumin for 45 min at 37° C (lane C). Lane D shows the electrophoretic mobility of the ^{125}I-labelled thrombin, which has not been incubated previously. The molecular weight markers given in kilodalton are 94, phosphorylase b; 67, bovine serum albumin; 43, ovalbumin. The stacking gel was 37.5 and the resolving gel 75 g/l of acrylamide. Details are given in Materials and Methods. *B.* Hepatocytes were incubated for 60 min at 37° C (●) or 90 min at 4° C (○) with ^{125}I-labelled thrombin as described above. The cells were concentrated by low speed centrifugation after a three-fold dilution with icecold buffer followed by centrifugation in a Beckman microfuge. The pellet was washed twice and dissolved in sodium dodecyl sulphate for electrophoresis. After sodium dodecyl sulphate electrophoresis on rod gels, the migration pattern was evaluated by counting 1.5 mm slices of the gel for ^{125}I activity

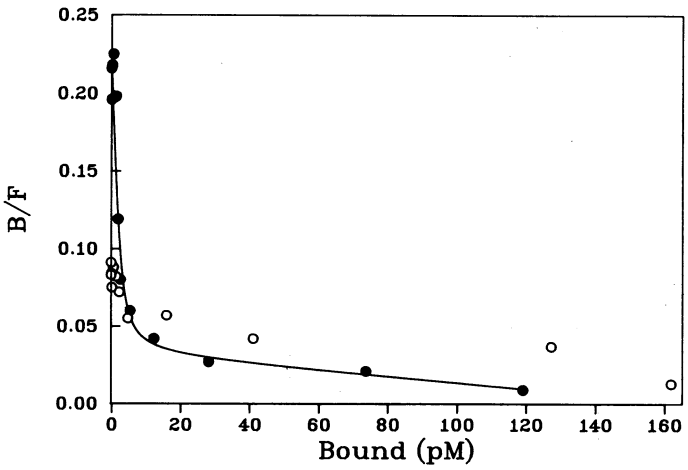

Fig. 7 The effect of heparin on the binding of ^{125}I-labelled thrombin to isolated rat hepatocytes. The different thrombin concentrations were preincubated for 30 min at 4° C in the absence (●) or presence (○) of 5 U/ml heparin. Isolated rat hepatocytes were added to give a final concentration of 1.6×10^6 cells/ml, and the incubation was continued for 90 min at 4° C. Other details were as described in the legend to Fig. 4. The data are plotted according to Scatchard after subtraction of the non-saturable binding (cf. Fig. 4). The curve represents the best computerized fit of a two compartment model to the data obtained in the absence of heparin

inhibitor hirudin, in a highly purified form, inhibited completely the binding of ^{125}I-labelled thrombin (Table 4). Heparin inhibited the binding to some extent (Table 4) due to an inhibition of the high affinity binding sites (Fig. 7). The binding was independent of divalent cations, since EDTA did not inhibit the binding (Table 4). Thus, in the isolated rat hepatocyte, the receptor recognition of the thrombin molecule is restricted to structures specific for thrombin.

Discussion

In addition to constituting the last part of the coagulation cascade, converting the soluble fibrinogen to the insoluble fibrin by limited proteolysis, thrombin has been shown to possess several other effects both inside and outside the coagulation system (for recent reviews, see ref. 40). A number of cell types possess receptors for thrombin. Thus, the platelet activation is the result of such a receptor binding (9–11). In addition, thrombin potentiates the growth-stimulatory effect of fibroblast growth factor on cultured human and bovine endothelial cells (41), and in fibroblasts, receptor-bound thrombin acts as a growth factor (12–14).

In view of the above mentioned results, and since both the production of thrombin's precursor, prothrombin (42), and the clearance of thrombin after its complex formation with antithrombin III (1) take place in the parenchymal liver cell, we found it relevant to look for the presence of specific thrombin receptors in the liver. We have used the isolated rat hepatocyte as a model, since this cell type is easily available and can be purified to homogeneity (21, 23, 24).

We find that the binding characteristics of the thrombin tracer was better after iodination with the chloramine-T than with the lactoperoxidase method (Table 1). By contrast, Machovich and coworkers (16, 43) found only a specific association of ^{125}I-labelled thrombin to isolated rat hepatocytes after iodination with lactoperoxidase (16), although not with exactly the same preparation of immobilized lactoperoxidase. However, the degree of iodination after their lactoperoxidase iodination was very low, one hundredth of that obtained by their chloramine-T catalyzed iodination (16, 43). Also, the binding kinetics reported by Machovich and coworkers deviate from the present results. Thus, they found no plateau in the uptake of 10 nM ^{125}I-labelled thrombin even after 3 h of incubation at 37° C (16), whereas our incubates reached a plateau within 20 min, even when using a 400-fold lower ligand concentration (Fig. 1).

The dissociation of radioactivity from the cells followed neither at 37° C (Fig. 3) nor at 4° C (Fig. 2) a monoexponential function. A fraction of the tracer remained "irreversibly" bound at both temperatures, when the dissociation took place in the absence of unlabelled thrombin (Table 2). Although there was no detectable decrease in the cell-associated radioactivity after the third hour of dissociation (unpublished data), it might represent a third compartment of very slowly dissociable thrombin. As cell death became significant at longer periods of incubation, the remaining cell-associated radioactivity was regarded as being irreversibly bound for these practical reasons. Irreversibly bound thrombin has also been observed in platelets (44), endothelial cells (45, 46), and fibroblasts (47).

The presence of unlabelled thrombin in the medium accelerated the dissociation of the bound ^{125}I-labelled thrombin threefold at 37° C (Fig. 3), and changed the distribution between the fractions dissociating fast and slowly (Table 2). This was not seen at 4° C (Fig. 2 and Table 2). If this is due to the expression of negative cooperativity, its temperature sensitivity is opposite to that of insulin, which shows increasing expression of negative cooperativity at decreasing temperatures (48, 49), but similar to that for angiotensin II binding to isolated rat glomeruli (50).

The binding affinities of thrombin reported here deviate from previously published studies on other cell types. This can of course be ascribed to the difference in tissue, but also to the use of lower concentrations of thrombin than those used in similar studies by other investigators. Employment of too high concentrations of ligand will lead to an underestimation of the affinity for the high-affinity sites. We find a K_d of 30–40 pM for the 4,500 high-affinity receptors and a K_d of 30 nM for the 2.8×10^5 low-affinity sites (Fig. 4). For the human platelet, the K_d for the high-affinity sites varies from 120 pM (51) to 3.2 nM (52), and the low-affinity sites from 4 nM (10) to 600 nM (52). Fibroblasts and endothelial cells have been reported to have receptors of a lower affinity (K_d = 0.7–3.0 nM for the high-affinity and 26–180 nM for the low-affinity) (13, 15, 53). Since the binding isotherm does not follow that predicted by a single class of independent binding sites at 4° C (Fig. 4), and negative cooperativity is not expressed at this temperature (Fig. 2), the presence of at least two independent classes of binding sites seems likely.

In other ligand-receptor systems clearly showing a receptor-mediated degradation, the fraction of endocytosed ligand is much higher than that seen with thrombin in hepatocytes (Fig. 5). Thus, for insulin the fraction of label resistant to treatment with pH 3 is about 0.8 in rat adipocytes (54) and HT-29 adenocarcinoma cells derived from human colon (55). For epidermal growth factor in Balb 3T3 cells, the similar figure is 0.85 (56). By contrast, after 20 min of incubation, 0.3 of the displaceably bound ^{125}I-labelled thrombin was resistant to treatment with buffer at pH 3, increasing to 0.4 after 60 min of incubation at 37° C (Fig. 5). In mouse embryo cells, the receptors were found in clusters unrelated to coated membrane areas, and no apparent receptor-mediated endocytosis was observed (57, 58). In chick embryo fibroblasts incubated for 10 h at 37° C with ^{125}I-labelled thrombin, 0.7 of the autoradiographic grains were localized intracellularly (59). Later studies have shown that endocytosis is almost exclusively mediated by the protease nexin system (60). The development of resistance to pH 3 and of irreversible binding are probably

unrelated, since the irreversibly bound fraction in the dissociation experiments was larger at 4° C than at 37° C (Table 2), whereas the opposite was found with the pH 3-resistance (Fig. 5).

Also the degradation of receptor-bound ligand was very small (Table 3) if present at all. Due to the proteolysis of the tracer even in the absence of cells, it is, however, difficult to quantitate in detail. When cells were incubated with ^{125}I-labelled thrombin, the degradation of the tracer in the cell suspension did not exceed that in a parallel incubation consisting of buffer and tracer without cells (unpublished observation). Of the tracer dissociating from the cells, about 0.75 retained full receptor binding capacity – a figure similar to that obtained for tracer incubated in a conditioned but cell-free medium (Table 3). More than 0.4 of the intracellular radioactivity in chick embryo fibroblasts incubated with ^{125}I-labelled thrombin had an electrophoretic mobility as native thrombin (59) and retained its esterolytic activity (61).

Incubation of ^{125}I-labelled thrombin in a conditioned medium from a cell suspension, which would bind 0.2 of the added tracer, did not give rise to any detectable radioactive band with a reduced mobility on a sodium dodecyl sulphate polyacrylamide gel electrophogram obtained under non-reducing conditions (Fig. 6A). Therefore, it is unlikely that the cells should have released, or that the incubation medium should have contained any protease inhibitor in a concentration sufficient to explain the binding data presented here. The former was also supported by the 4° C experiments, where secretion should be minimal. Only one isotopic species was found when the cell-associated radioactivity was subjected to sodium dodecyl sulphate gel electrophoresis. This migrated as iodo-thrombin (Fig. 6B). Also the low rates of receptor-mediated endocytosis and degradation exclude that a binding to one of the scavenger binding mechanisms constitutes a quantitatively important part of the cellular uptake of thrombin (1, 60). Thus, it seems very unlikely that the binding should occur to receptors for antithrombin III (1, 62) and protease nexins (60, 63). The receptors for protease complexes with α_2-macroglobulin (6) and α_1-proteinase inhibitor (7, 8) were excluded experimentally (Table 4), and all these uptake systems should have been saturated after the addition of a surplus of urokinase and elastase. This was not the case (Table 4).

Heparin accelerates the rate of linkage between soluble protease nexin and thrombin (63), but the cellular binding of the complex is inhibited in the presence of heparin (60). Heparin has been shown to reduce the binding of thrombin to rabbit aorta endothelial cells (53) and to human polymorphonuclear leukocytes (Sonne, unpublished observation). In platelets the decreased binding of thrombin in the presence of heparin occurred through a reduction of the binding to the high-affinity binding sites (64), as also found in the present system (Fig. 7).

The receptor recognizes a specific determinant present in the thrombin molecule, since 150 nM prothrombin and prethrombin 1 did not displace ^{125}I-labelled thrombin (Table 4). However, a conformational change of the prethrombin 1 induced by cleaving the peptide bond Arg^{323}-Ile^{324} by small amounts of ecarin, resulting in the formation of meizothrombin 1, introduced the receptor recognition site (Table 4). It is therefore unlikely that thrombin should be bound to one of the lectins of the liver through its glycosylation side chains. This was also directly shown by the lack of interference with the binding of thrombin by 50 mM of either of the sugars *N*-acetylglucosamine, D(+)galactose, D(+)mannose, α-D(+)fucose, D-fructose, or D(+)glucose (Sonne, unpublished observation). Since also the thrombin-specific inhibitor hirudin (65) inhibited the binding, the receptor seems to be highly specific for a structure, which is only exposed in the thrombin molecule. The esterolytic inhibitors phenylmethanesulphonyl fluoride and benzamidine did not inhibit the binding (Table 4). When a preparation of ^{125}I-labelled thrombin, of which >0.85 of the radioactivity reacted with antithrombin III in the presence of heparin, was compared with tracer from the same batch but treated with diisopropyl fluorophosphate so that <0.04 reacted with antithrombin III, exactly the same time courses of dissociation including identical fractions of irreversibly bound radioactivity were achieved at both 4 and 37° C (Pedersen K, Fisker S, Kudahl K, Sonne O, unpublished observations). This suggests that the receptor recognition site is most likely outside this region but involving a region covered by hirudin (Table 4).

In conclusion, hepatocytes possess specific binding sites for thrombin. The heterogeneous pool of receptors consists of a few thousand high-affinity sites and about 3×10^5 low-affinity sites per cell. A physiological role, except for an expansion of the distribution space for thrombin, should be mediated via a high-affinity site, since the concentration of free thrombin is low due to the presence of a high protease inhibitory capacity of the plasma proteins.

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