Volume 334, number 2, 225–228 FEBS 13245 November 1993

Long-term effects of thrombin require sustained activation of the functional thrombin receptor

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Received 8 September 1993

Thrombin is a potent activator of human glomerular epithelial cells (HGEC). Here we compare short-term and long-term effects of thrombin and thrombin receptor agonist peptide (TRAP) which selectively activates the functional thrombin receptor. TRAP, as thrombin, increases intracellular free Ca^{2+} concentration and acts synergistically with growth factors possessing tyrosine kinase receptors on DNA synthesis. Thrombin induces synthesis of proteins of the fibrinolytic system and cell proliferation if it is present for at least 8 h. TRAP alone does not stimulate protein synthesis and is not mitogenic. However, in the presence of the aminopeptidase inhibitor amastatin all long-term effects of thrombin can be fully mimicked by TRAP. In conclusion, different effects of thrombin and TRAP may be related to the degradation of TRAP by cellular ectoenzymes. The recently cloned thrombin receptor accounts for early intracellular signals and long-term cellular effects that require sustained activation of this receptor.

Thrombin; Thrombin receptor; TRAP; Aminopeptidase; Glomerular epithelial cell

1. INTRODUCTION

Human extracapillary glomerulonephritis is characterized by glomerular fibrin deposition and proliferation of HGEC, possibly mediated by thrombin [1]. In addition to its critical role in thrombosis and haemostasis, thrombin exhibits multiple cell-activating functions [2,3]. Thrombin is a potent platelet agonist [4], chemotactic for monocytes [5], mitogenic for mesenchymal cells [6] and activates endothelial cells [7]. The effects of thrombin on HGEC and signal transduction mechanisms involved have previously been described [8,9].

Recently, a functional receptor of thrombin has been cloned [10,11]. The thrombin receptor is a member of the multigene family of seven transmembrane domain receptors, couples to G_q-like and G_i-like guanine nucleotide-binding proteins (G-proteins) and stimulates phosphoinositide hydrolysis and inhibition of adenylate cyclase [12,13].

Receptor activation occurs by cleavage of the N-terminal extracellular domain of the receptor by thrombin. The new N-terminus exposed acts as a tethered peptide ligand and activates the receptor [10,11]. Accordingly, receptor activation can be mimicked by synthetic peptides corresponding to the new N-terminus (i.e. SFLLRNP = TRAP) [14].

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Abbrevations: HGEC, human glomerular epithelial cells; TRAP, thrombin receptor agonist peptide; u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor.

Whether the cloned functional thrombin receptor is responsible for all cellular effects of thrombin remains controversial. In contrast to thrombin, TRAP does not mediate the second phase of p44 mitogen-activated protein kinase activation and DNA synthesis reinitiation in CCL39 fibroblasts, whereas it is as competent as thrombin in activating phospholipase C and in inhibiting adenylate cyclase [15,16]. In endothelial cells, TRAP induces an increase in intracellular free Ca^{2+} concentration and expression of P-selectin, but not expression of intracellular adhesion molecule 1, increase in transendothelial permeability and translocation of protein kinase C that are stimulated by thrombin [17,18].

Recently, Coller et al. have demonstrated that aminopeptidase M in plasma and on endothelial cells cleaves and thereby inactivates TRAP [19]. Accordingly, inhibition of aminopeptidase M enhances platelet aggregation induced by TRAP pointing to the possibility of rapid degradation of TRAP in in vivo and in vitro systems.

Here we compare the cellular effects of thrombin and TRAP to determine the role of the functional thrombin receptor in HGEC.

2. MATERIALS AND METHODS

2.1. *Materials*

The following materials have been obtained as indicated: purified human α-thrombin (3000 U/mg, 1 U/ml = 10^{-8} M), collagenase type IV, hirudin, amastatin, bestatin, thiorphan from Sigma; polyclonal antibodies to human tissue-type plasminogen activator (t-PA) and to human urokinase-type plasminogen activator (u-PA), polyclonal and monoclonal (MAI 12) antibodies to plasminogen activator inhibitor 1 (PAI-1) from Biopool; ionomycin, fura-2 acetoxymethylester from Calbiochem; epidermal growth factor (EGF) and [^{3}H]thymidine (20 Ci/mmol) from Amersham; basic fibroblast growth factor (bFGF)

from Choay, Paris. TRAP (SFLLRNP) was a generous gift from Dr. Seiler, Bristol-Squibb, Princeton.

2.2. *Cell culture*

Glomerular epithelial cells were obtained by collagenase digestion of isolated glomeruli derived from normal human kidneys judged to be unsuitable for transplantation. They were purified using cloning rings and identified by their morphologic and functional features as previously described [20].

Incubations were performed on the third subculture of HGEC grown in multidish wells (Nunclon, Nunc) in normally defined medium as described [8]. Before reaching confluence, cells were washed, growth arrested by serum starvation for 24 h and incubated with the compound to be tested for 24 h. Culture media were collected for determination of fibrinolytic components.

2.3. *Measurement of DNA synthesis*

Six hours before the end of incubation, 1 μCi [^{3}H]thymidine was added to each well. Cells were washed, incubated 30 min in minimum defined medium containing 1 mg/ml non-labeled thymidine and then harvested by trypsinization. Cells were filtered on 0.45 μm Millipore filter and the radioactivity of the filter was counted in a β-scintillation counter.

2.4. *Determination of cytosolic free calcium concentration* $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in HGEC was measured using fura-2 acetoxymethylester (4 μM) exactly as described [9]. Fluorescence was monitored continuously in a Perkin-Elmer model LS-5 spectrofluorometer using 339 nm excitation/500 nm emission wavelengths. Calibration of the Ca^{2+}-dependent fluorescence was obtained by addition of 20 μM ionomycin (maximum fluorescence, F_{max}) followed by 4 mM EGTA in 60 mM Tris-HCl buffer, pH 10.5 (minimal fluorescence, F_{min}). $[Ca^{2+}]_i$ was calculated using the following formule:
$[Ca^{2+}]_i = (F - F_{min} \times K_d/(F_{max} - F)$ with $K_d = 224$ nM.

2.5. *Enzyme linked immunosorbent assay for t-PA, u-PA and PAI-1*

Determination of u-PA, t-PA and PAI-1 antigen were performed as previously described [8]. The detection limits for PAI-1, u-PA, and t-PA were 0.4 ng/ml, 0.9 ng/ml, and 0.14 ng/ml, respectively.

3. RESULTS

3.1. *Effect of thrombin and TRAP on early transmembrane signals*

As shown in Fig. 1, thrombin and TRAP induce a rapid and transient increase in intracellular free Ca^{2+} concentration which was dose-dependent. Maximal responses by saturing concentrations of TRAP (100 μM) and thrombin (80 nM) are similar. The aminopeptidase inhibitor amastatin has no effect on thrombin and TRAP induced Ca^{2+} dependent fluorescence (not shown).

In parallel, TRAP is as potent as thrombin in stimulating GTPase activity and generation of inositol-trisphosphate (manuscript in preparation).

3.2. *Long-term effects of thrombin and TRAP*

It has previously been shown that thrombin is mitogenic for HGEC [8]. Thrombin induces a dose-dependent increase in DNA synthesis in HGEC (Fig. 2A). The continuous presence of thrombin for at least 8 h is required to induce cell-cycle re-entry. TRAP alone has no mitogenic effect (Fig. 2B). However, in the presence of the aminopeptidase inhibitor amastatin, TRAP is also able to stimulate DNA synthesis. Amastatin is the most effective inhibitor tested so far. Another aminopeptidase inhibitor, bestatin (up to 100 μM) is only partially effective in protecting TRAP from degradation. Thiorphan (up to 100 μM), an inhibitor of neutral endopeptidase [21], has no effect. In contrast, amastatin does not influence thrombin induced mitogenesis (Fig. 2A).

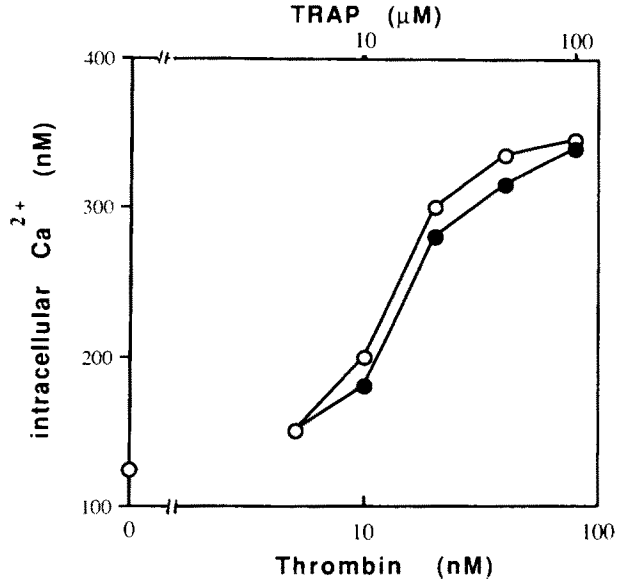

Fig. 1. Increase in intracellular free Ca^{2+} concentration. Dose–response effect of thrombin (○) and TRAP (●) on fura-2 fluorescence. Means of 5 experiments are presented.

HGEC in culture synthesize u-PA, t-PA and PAI-1. It has previously been shown [8] that thrombin induces a 2–3-fold increase in u-PA, t-PA and PAI-1 antigen release in the supernatant during 24 h (Fig. 3). TRAP alone has no effect. In the presence of amastatin, however, TRAP is as potent as thrombin in inducing protein synthesis (Fig. 3).

The relative potencies of TRAP and thrombin were similar for long- and short-term effects. The higher potency of thrombin compared to TRAP is consistent with the proposed model of receptor activation in which thrombin acts enzymatically whereas TRAP acts by binding.

3.3. *Synergism between thrombin and growth factors possessing tyrosine kinase receptors*

Growth factors, activating receptor associated G-proteins or receptor tyrosine kinases can act synergistically on DNA synthesis by stimulation of different intracellular pathways [22]. For example, thrombin potentiates DNA synthesis in cooperation with EGF and bFGF [15,23], platelet-derived growth factor and insulin [24].

Thrombin-induced mitogenesis is totally blocked by addition of hirudin, a potent inhibitor of thrombin [25], one hour after thrombin. However, a synergistic effect

with EGF and bFGF is still observed (Table I). This suggests that a transient phase of activation induced by thrombin during one hour is sufficient for a synergistic action with other growth factors, but not enough to induce mitogenesis by itself. Accordingly, a synergistic effect with EGF and bFGF can also be mimicked by TRAP in the absence of aminopeptidase inhibitors (Table I).

4. DISCUSSION

Both thrombin and TRAP induce G-protein-coupled early intracellular signals. Here the increase in intracellular free Ca^{2+} concentration is shown as an example. In contrast, TRAP can mimick long-term effects of thrombin as induction of cell proliferation and protein synthesis only in the presence of aminopeptidase inhibitors.

HGEC are rich in cell surface associated proteolytic enzymes. Ectoenzymes like aminopeptidase-A, aminopeptidase-N and neutral endopeptidase are present and responsible for proteolytic activation/inactivation of bioactive peptides [26]. Aminopeptidase-N-like activity on HGEC has been detected and could be inhibited by amastatin [27] suggesting rapid degradation of TRAP by aminopeptidase-N.

Degradation of TRAP may be one explanation for the lack of medium- and long-term effects of TRAP in some cell systems and may depend finally on the cellular equipment with proteolytic enzymes. The sustained activation of the functional thrombin receptor is required to observe long-term cellular effects. This is indicated by the lack of effect of TRAP alone, which is rapidly degraded, on protein synthesis and cell proliferation and by the inhibition of thrombin effects when hirudin is added up to 8 h after thrombin.

In contrast, aminopeptidase inhibitors do not influence thrombin-induced cellular effects. Therefore the aminopeptidase degrading TRAP does not appear to

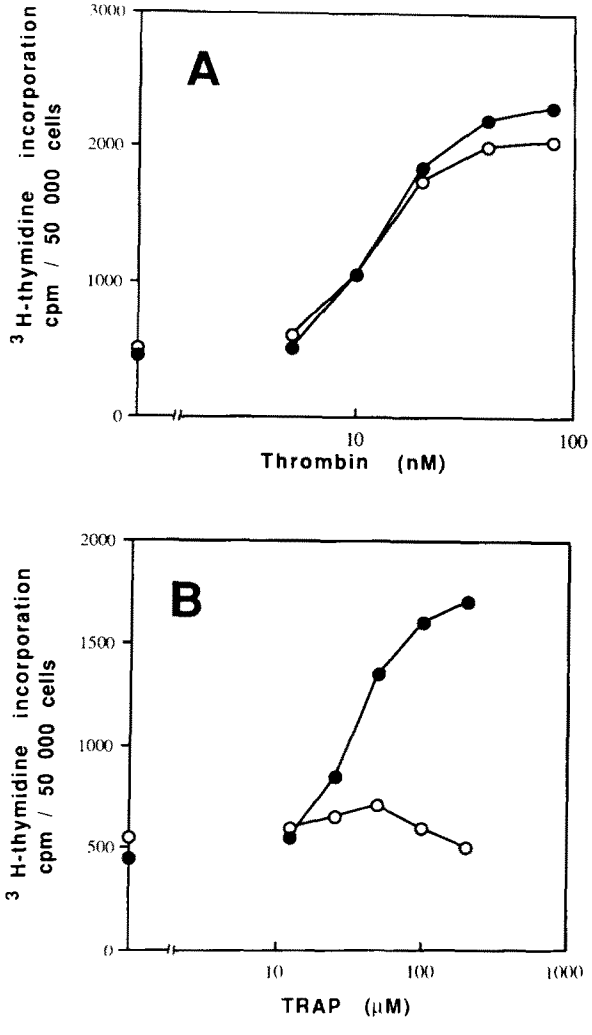

Fig. 2. Stimulation of DNA synthesis. Dose–response effects of thrombin (A) and TRAP (B) on [^{3}H]thymidine incorporation in the presence (●) or absence (○) of amastatin (10 μM). Means of 3 experiments made in triplicate are presented.

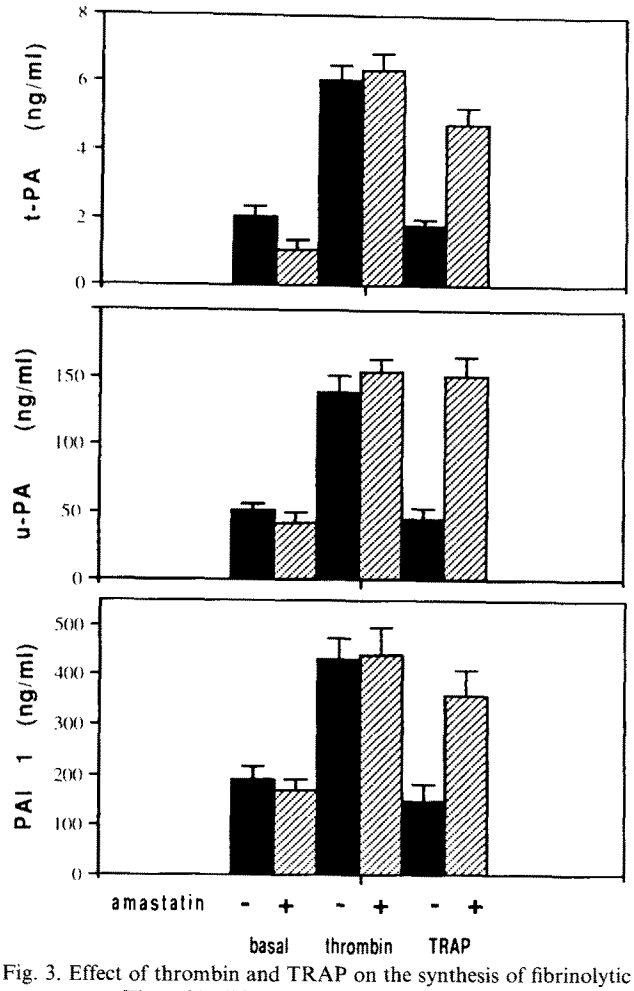

Fig. 3. Effect of thrombin and TRAP on the synthesis of fibrinolytic components. Thrombin (20 nM) or TRAP (50 μM) were incubated in the presence or absence of amastatin (10 μM) and the concentration of u-PA, t-PA and PAI-1 antigen in the conditioned media were measured. Means ± S.E.M. of four different experiments made in triplicate are presented.

Table I

Effect of growth factors, thrombin and TRAP on [^{3}H]thymidine incorporation in HGEC

Culture treatment	[^{3}H]Thymidine incorporation (cpm/5 × 10^4 cells)		
	–	+ EGF (10 ng/ml)	+ bFGF (50 ng/ml)
Basal	461 ± 55	1282 ± 146	877 ± 51
Thrombin (20 nM)	1489 ± 201	2995 ± 264	2267 ± 163
Thrombin (20 nM) + hirudin (2 μM)*	451 ± 60	2141 ± 315	1443 ± 145
TRAP (50 μM)	493 ± 46	2105 ± 234	1367 ± 114

Means ± S.E.M. of 4 different experiments made in triplicate are presented.
*Hirudin was added one hour after thrombin.

inactivate the tethered ligand produced by thrombin cleavage of its receptor under the experimental conditions used. This is in good agreement with results of Coller et al. [19] on platelet aggregation.

In conclusion, our results suggest that the recently cloned functional thrombin receptor mediates early intracellular signals and long-term effects of thrombin on HGEC. Induction of long-term effects requires sustained activation of this receptor by thrombin.

Acknowledgements: U.Z. was a recipient of a fellowship for the French Ministry of Foreign Affairs. This work was supported in part by grants from the Institut National de la Santé et de la Recherche Médicale and from the Association Claude Bernard. We thank Dr S. Seiler (Bristol-Myers-Squibb, Princeton) for his generous gift of TRAP.

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