# Plasmin modulates the thrombin-evoked calcium response in C6 glioma cells

Joy S. TURNER,* Gerard T. REDPATH,* John E. HUMPHRIES,† Steven L. GONIAS*‡ and Scott R. VANDENBERG*§

Departments of *Pathology (Neuropathology), †Internal Medicine, and ‡Biochemistry, University of Virginia Health Sciences Center, Charlottesville, VA 22908, U.S.A.

Extracellular proteinases may be selectively targeted to cell surfaces by specific receptors or binding sites. In previous studies, we have characterized cellular binding sites for plasminogen and plasmin on rat C6 glioma cells. In this investigation, we studied the response of C6 cells to α-thrombin and plasmin by measuring the rapid kinetics of free intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$). Thrombin produced a strong, concentration-dependent rise in $[Ca^{2+}]_i$ with an onset within 3 s and peak levels achieved in less than 10 s. A similar response was also evoked by an SFLLRN-containing thrombin-agonist peptide. C6 cells did not respond to plasmin (25 nM–1.5 μM). By contrast, pretreatment of C6 cells with 100 nM plasmin significantly inhibited the $[Ca^{2+}]_i$ response to thrombin and the thrombin-agonist peptide. The peak $[Ca^{2+}]_i$ response to thrombin, in cells pretreated with plasmin, was reduced by approx. 50%. The effect of plasmin on the cellular response to thrombin was selective, as pretreatment of the cells with plasmin did not affect the $[Ca^{2+}]_i$ response to platelet-activating factor. Di-isopropylphosphorylplasmin and plasminogen did not inhibit the cellular response to thrombin, indicating that plasmin activity is required and that occupancy of cellular plasmin(ogen)-binding sites alone is insufficient. These studies demonstrate that plasmin does not directly induce a response in C6 cells, but may affect cellular function by specifically modulating the thrombin response.

## INTRODUCTION

α-Thrombin binds to a wide variety of cell types and evokes numerous responses, most of which are associated with cellular activation [1–3]. A thrombin receptor has been cloned and sequenced using cDNA from different cell types; in each case the same protein has been identified [1,3–5]. The cloned thrombin receptor has an apparent protein mass of 47 kDa, is significantly N-glycosylated [5], has seven transmembrane domains, and is coupled via G-proteins to a variety of signal transduction systems [1,5]. Comparison of the cDNA sequences of the human and rat thrombin receptors indicates that the receptors are 78% similar at the amino acid level [6].

Like thrombin, plasmin associates with a specific and saturable system of binding sites in a variety of cell types [7]. The affinity of the plasmin-binding interaction is low (0.1–1.0 μM) but the cellular binding capacity is typically high ($10^5$–$10^7$ sites per cell). Plasmin and the zymogen [Glu$^1$]plasminogen bind to the same cellular sites; the interaction is dependent upon a series of kringle domains which express lysine-binding sites [8].

The molecular nature of cellular plasmin(ogen)-binding sites remains incompletely understood and, in fact, a variety of plasma membrane macromolecules may contribute to the total cellular plasminogen-binding capacity. Nevertheless, sequestration and concentration of plasminogen at the cell surface is important for a variety of proteinase-mediated processes that involve cellular migration or tissue remodelling [9]. While associated with the cell surface, plasmin demonstrates significant proteolytic activity towards a variety of substrates and is relatively resistant to inhibition by proteinase inhibitors such as $\alpha_2$-antiplasmin and $\alpha_2$-macroglobulin [10]. Therefore, bound plasmin may selectively alter substrates in the microenvironment of the cell surface, including integral plasma membrane proteins. Although the effects of plasmin and plasminogen on cellular signal transduction are largely uncharacterized, it was recently reported that plasmin binding to human umbilical vein endothelial cells inhibits protein kinase activity [11].

Platelet-activating factor (PAF) is produced by two independent enzymic pathways involving either structural modification of a membrane lipid or *de novo* synthesis from an *O*-alkyl analogue of a lysophosphatidic acid [12]. As a potent lipid mediator of cellular function, PAF induces a wide range of biological responses including activation of protein kinases, and stimulation of arachidonic acid and phosphoinositide metabolism [12–15]. PAF interacts through the *sn*-2 position of its acetyl moiety with a surface membrane receptor that is proteinase sensitive and appears to be coupled to a G-protein [12,14–16]. In the brain, PAF functions in ischaemic and traumatic injury, exerting cytotoxic effects on neuronal cells, causing vasoconstriction and modulating the blood–brain barrier [17,18].

Previous studies have demonstrated that rat C6 glioma cells have specific cellular binding sites for plasminogen and plasmin [19]. Furthermore, these cells respond to thrombin with transitions of free intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) [20] and to PAF with increased astrocytic differentiation [21]. Thus, C6 cells represent a suitable model in which to examine the effects of plasmin on specific receptor-mediated cellular responses. The first goal of this investigation was to determine whether plasmin (or plasminogen) binding to C6 cells results in a $[Ca^{2+}]_i$ transient. The second goal was to determine whether

Abbreviations used: $PAF_{16}$, platelet activating factor (L-α-phosphatidylcholine, β-acetyl-γ-*o*-hexadecyl); PPACK, D-phenylalanyl-L-prolyl-L-arginylchloromethyl ketone; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; DIP-plasmin, di-isopropylphosphoryl plasmin; Indo-1 AM, indo-1 acetoxymethyl ester; S-2251, D-Val-L-Leu-L-Lys-*p*-nitroanilide hydrochloride; DFP, di-isopropylphosphorofluoridate; SEBS, Earle's balanced salts solution supplemented with 5.5 mM *myo*-inositol, 27.7 mM glucose, 5 mM sodium pyruvate, 0.76 mM serine and 40 μM glycine at pH 7.4.

§ To whom correspondence should be addressed.

plasmin might selectively alter the response of C6 cells to thrombin or $PAF_{16}$.

## EXPERIMENTAL

### Proteins and peptides

Bovine α-thrombin was obtained from Sigma. Thrombin was inactivated by incubation with 0.1 μM D-Phe-Pro-Arg-$CH_2Cl$ (PPACK) for 10 min. [Glu¹]Plasminogen was purified from fresh frozen human plasma as previously described [22] and dialysed against 10 mM Hepes/50 mM sodium acetate, pH 7.4. Plasminogen (3.0–5.0 μM) was activated with low-molecular-mass urokinase (20 nM) (Calbiochem) for 15 min at 37 °C. The extent of activation was determined by active-site titration with *p*-nitrophenyl *p*-guanidinobenzoate [23] or by the rate of hydrolysis of the plasmin-specific substrate, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide hydrochloride (Kabi Vitrum), using previously determined kinetic parameters [24]. Di-isopropylphosphoryl plasmin (DIP-plasmin) was prepared by incubating plasmin twice with 10 mM di-isopropylphosphorofluoridate (DFP; Sigma) for 1 h at 22 °C. Excess DFP was removed by dialysis against 20 mM sodium phosphate/154 mM NaCl, pH 7.4. Residual plasmin activity in DIP-plasmin preparations was always less than 0.1 % as determined by S-2251 hydrolysis.

The thrombin-receptor tethered ligand peptide, SFLLRNPNDKYEPFC, was synthesized by the Protein and Nucleic Acids Core Laboratory at the University of Virginia. The structure was confirmed by amino acid analysis and mass spectroscopy. The first five or six residues of this peptide constitute the essential sequence for thrombin-receptor activation [25,26].

### Cell culture

C6 glioma cells were obtained from the American Type Culture Collection and were regularly determined to be free of mycoplasma contamination (Gen-Probe, Inc., San Diego, CA, U.S.A.). The growth medium was α-Minimal Essential Medium (GIBCO, Gaithersburg, MD, U.S.A.), 10 % (v/v) fetal bovine serum (A-1111-D, Hyclone, Logan, UT, U.S.A.) and 20 μg/ml bovine zinc–insulin (25.7 i.u./mg; Sigma, St. Louis, MO, U.S.A.). The cells were passaged after reaching 90 % confluency (every 4–5 days) and plated at an initial density of (6–8) × $10^3$ cells/$cm^2$ at 37 °C, in 4.8 % $CO_2$, at 90 % relative humidity.

Experiments were performed with cell suspensions obtained from 90 % confluent monolayers. The growth medium was replaced with saline buffer [137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.4 mM $NaH_2PO_4$, 0.035 % (w/v) BSA, 0.4 % (w/v) glucose and 0.5 mM EGTA] and the cells were released by gentle agitation at 37 °C (5–10 min). The resulting suspensions were diluted into a 4-fold vol. of Earle's Balanced Salts solution supplemented with *myo*-inositol (5.5 mM), glucose (27.7 mM), sodium pyruvate (5 mM), serine (0.76 mM) and glycine (40 μM), pH 7.4 (SEBS) and washed by centrifugation at 160 *g* for 7 min.

### $[Ca^{2+}]_i$ determination by rapid stopped-flow and slow kinetics

C6-cell suspensions were washed and resuspended in Leibovitz's L-15 medium, containing 2 % (v/v) fetal bovine serum, pH 7.4. Indo-1 acetoxymethyl ester (Indo-1 AM) (Molecular Probes) was added to the suspensions at a final concentration of 1.5 μM and the cells were incubated for 25 min at 37 °C. The cells were then washed by centrifugation to remove free Indo-1 AM, resuspended in SEBS solution at 22 °C and used immediately to measure calcium responses. Fluorimetric determinations (1000 points for each response) were made as emission ratios at 398/480 nm using an excitation wavelength of 340 nm.

For rapid stopped-flow fluorescence measurements, a water-jacketed HiTec SFA-11 Rapid Kinetics Accessory (0.5 ml quartz cuvette, 1 cm light path) (Hi-Tech Scientific, Salisbury, U.K.) was coupled to an SLM 8000 spectrofluorimeter (SLM-AMINCO). The C6 cells were loaded into one channel and thrombin or the thrombin-agonist peptide was loaded into the second channel. The solutions were allowed to warm to 37 °C. Rapid mixing of the cells with the agonists occurred in the cuvette with an approximate dead time of 30 ms.

For measuring PAF effects, and other C6-cell responses which required longer than 30 s, the cells were loaded into a conventional cuvette (1 ml, 1 cm path length) with gentle stirring and the drug solution was added after establishing a baseline for 15–50 s. Emission ratios were obtained at 0.2 s intervals. The response of C6 cells to plasmin alone was measured by both rapid and slow techniques. To examine the effects of plasmin on thrombin- or PAF-evoked responses, the cells were incubated with 100 nM plasmin at 22 °C for 5 min and then immediately studied in either the stopped-flow or slow kinetics mode.

The fluorescence emission ratios were converted into $[Ca^{2+}]_i$ according to the method of Grynkiewicz et al. [27]. For each preparation of Indo-1 loaded cells, the maximum $[Ca^{2+}]_i$ value ($R_{max.}$) [27] was determined by treating the cells with 2.0 μM ionomycin (Molecular Probes). $R_{min.}$ [27] was determined by treating cells with ionomycin followed by 10 mM EGTA, at values at or above pH 8.0 for 20 min.

## RESULTS

### Thrombin-receptor-mediated $[Ca^{2+}]_i$ responses

Indo-1-loaded rat C6 glioma cells, which were dissociated from monolayer cultures, demonstrated a resting $[Ca^{2+}]_i$ value of

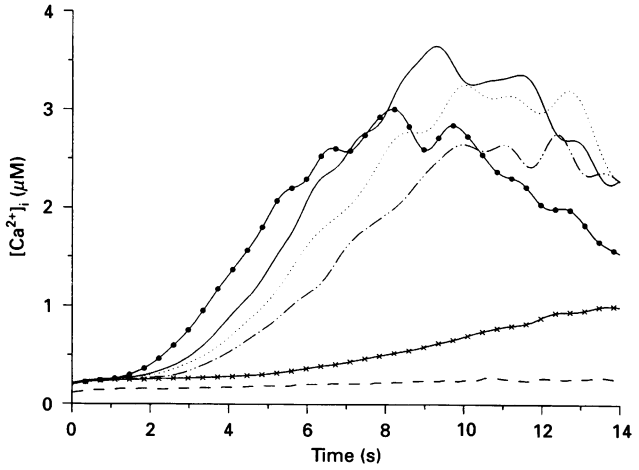


**Figure 1 C6 cellular response to α-thrombin**

Increasing concentrations of α-thrombin evoked both shorter latencies and higher amplitudes of $[Ca^{2+}]_i$, using stopped-flow analysis with Indo-1-loaded cells. All curves represent smoothed averages of three data sets of 1000 points. Thrombin concns.: 8 nM (●); 4 nM (——); 2.5 nM (· · ·); 0.8 nM (–· ·–); 0.4 nM (×); 8 nM thrombin pretreated with 0.1 μM PPACK (– –).

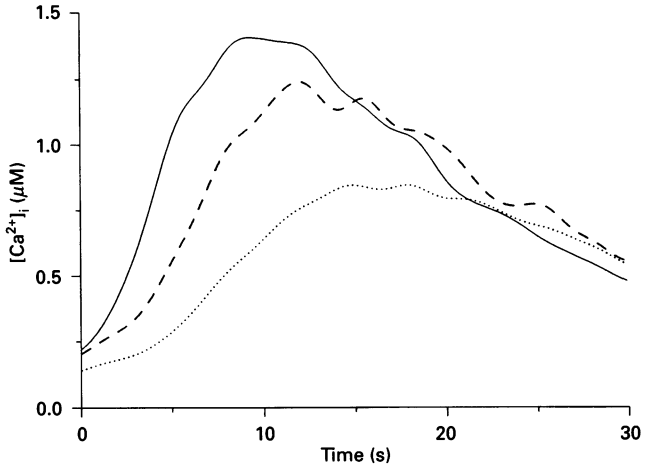


**Figure 2 C6 cellular response to thrombin-agonist peptide**

Increasing concentrations of SFLLR agonist peptide evoked $[Ca^{2+}]_i$ responses with similar latencies and durations as α-thrombin. Stopped-flow analysis; curves represent smoothed averages of three data sets of 1000 points. 60 μg/ml peptide (solid line); 40 μg/ml peptide (broken line); 20 μg/ml peptide (dotted line).

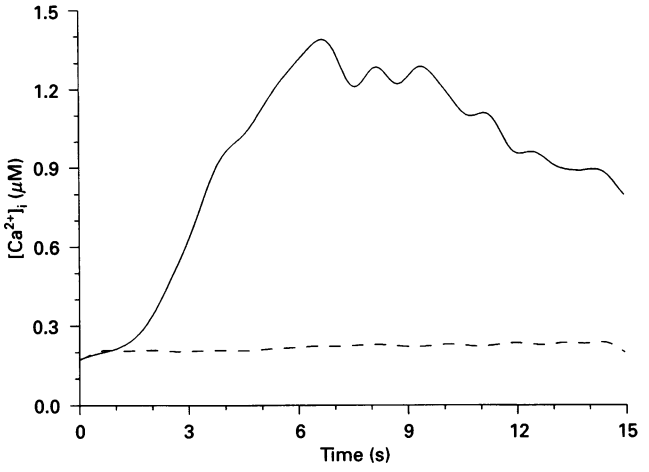


**Figure 3 Comparative responses of C6 cells to α-thrombin and active plasmin**

α-Thrombin (16 nM; solid line) evoked a marked and rapid $[Ca^{2+}]_i$ response while active plasmin (100 nM) alone (broken line) failed to change resting $[Ca^{2+}]_i$ levels. Stopped-flow analysis; all curves represent smoothed averages of three data sets of 1000 points.

195 ± 26 nM. This level varied less than 5% during a typical 1 h assay in SEBS solution at 22 °C. Leakage of the free acid form of Indo-1 from the cells was negligible.

Thrombin evoked an increase in free $[Ca^{2+}]_i$ and the magnitude of the effect was thrombin-concentration dependent. The maximum amplitude of response was observed with 2–4 nM thrombin (Figure 1). The $[Ca^{2+}]_i$ response was rapid, with an onset within 3 s, and transient, with $[Ca^{2+}]_i$ levels returning to resting values within 30–40 s. Although concentrations of thrombin higher than 4 nM shortened the latency of response, the amplitude and duration of response were either unchanged or decreased. When

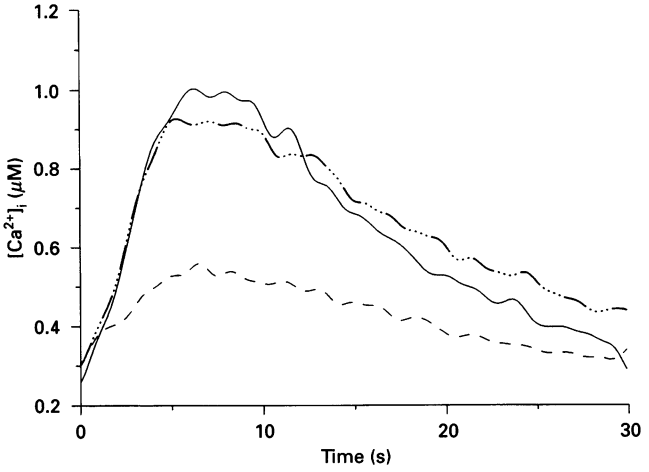


**Figure 4 Plasmin inhibition of the C6-cell response to α-thrombin**

Pretreatment of C6 cells with plasmin (100 nM) for 5 min at 22 °C significantly diminished the amplitude of the rapid α-thrombin response. Note the lack of effect with inactive DIP-plasmin (100 nM). Stopped-flow analysis: curves represent smoothed averages of three data sets of 1000 points. 8 nM α-thrombin (——); 8 nM α-thrombin after pretreatment with 100 nM DIP-plasmin (—·····—); 8 nM α-thrombin after pretreatment with 100 nM plasmin (—–—).

thrombin was inactivated by pretreatment with PPACK, the $[Ca^{2+}]_i$ response was completely abolished. The thrombin-agonist peptide also induced a marked increase in $[Ca^{2+}]_i$ in C6 cells (Figure 2). The latency and duration of the response were comparable with those observed with thrombin.

## Plasmin effects on $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ levels did not change in C6 cells which were exposed to plasminogen or plasmin (10–100 nM) for up to 5 min (Figure 3). During the monitoring period, cellular aggregation was not observed. Higher concentrations of plasmin were not typically studied as it has been previously shown that C6 cells tolerate plasmin at levels up to 100 nM without signs of altered viability or morphology [19]. Nevertheless, in order to determine whether plasmin activates the thrombin receptor with low efficiency, we assessed $[Ca^{2+}]_i$ levels in cells treated with 0.2–1.5 μM plasmin. Significant changes in $[Ca^{2+}]_i$ were not observed within 5 min.

## Plasmin modification of thrombin and agonist-peptide responses

Pretreatment of C6 cells with plasmin (100 nM) for 5 min reduced the overall amplitude of the $[Ca^{2+}]_i$ response to thrombin (8 nM) by 50% (Figure 4) and to the thrombin-agonist peptide (20 μg/ml) by 40% (Figure 5). When the concentration of thrombin was increased to 40 nM, the response in plasmin-pretreated cells was only slightly improved (less than 10%). Similarly, increasing the concentration of agonist peptide to 60 μg/ml improved the response by only 6% (compared with control cells which were not pretreated with plasmin). The latency and duration of the response to thrombin and agonist peptide were essentially unchanged in plasmin-treated cells. C6 cells that were pretreated with DIP-plasmin (Figure 4) or plasminogen (results not shown) demonstrated unchanged responses to thrombin. Thus, plasmin activity is necessary to alter the C6-cell thrombin-receptor response.

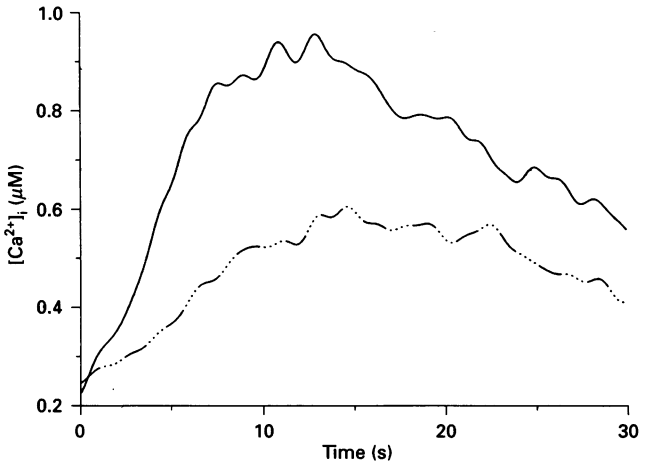


**Figure 5 Plasmin inhibition of the C6-cell response to thrombin-agonist peptide**

Pretreatment of the cells with active plasmin (100 nM) for 5 min at 22 °C significantly diminished the amplitude of the $[Ca^{2+}]_i$ response to thrombin-agonist peptide. Stopped-flow analysis; all curves represent smoothed averages of three data sets of 1000 points. 20 μg/ml SFLLRNPNDKYEPFC (——); 20 μg/ml peptide after plasmin pretreatment (–· · · ·–).

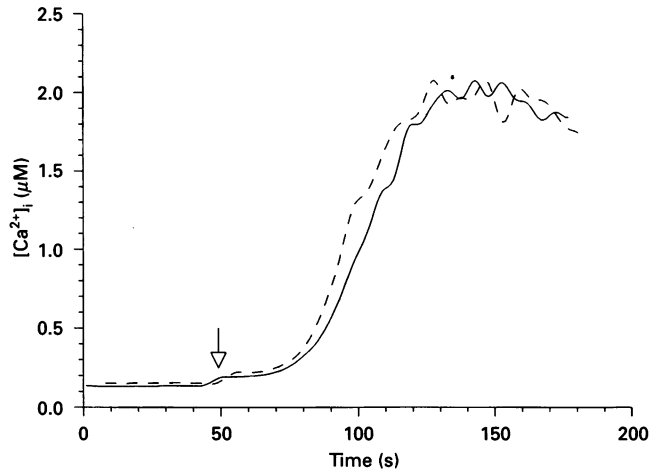


**Figure 6 Absence of plasmin effects on the C6 cellular response to $PAF_{16}$**

The $[Ca^{2+}]_i$ response to treatment of C6 cells with $PAF_{16}$ was significantly slower with more sustained maximum levels than with α-thrombin. Pretreatment of the cells with active plasmin (100 nM) for 5 min at 22 °C did not alter the $PAF_{16}$ response. $PAF_{16}$ was added (arrow) to gently stirred cells (30 rev./min). Slow kinetics analysis; all curves represent smoothed averages of two or three sets of 900 points. 10 μM $PAF_{16}$ (solid line); 10 μM $PAF_{16}$ after pretreatment with 100 nM plasmin (broken line).

### PAF-receptor-mediated $[Ca^{2+}]_i$ responses

C6-cell $[Ca^{2+}]_i$ levels increased in response to $PAF_{16}$ (Figure 6) and the magnitude of the effect was $PAF_{16}$-concentration dependent (results not shown). The minimum $PAF_{16}$ concentration which induced a response over resting levels was 5 nM and the maximum $[Ca^{2+}]_i$ response occurred with 10 μM $PAF_{16}$. The time course of free $[Ca^{2+}]_i$ changes in response to $PAF_{16}$ differed significantly from that observed with thrombin. The onset of the response started 20 s after adding the $PAF_{16}$ and the peak amplitude occurred after 90 s. The $[Ca^{2+}]_i$ levels did not return to resting levels within 150 s, which was the longest interval measured. Pretreatment of C6 cells with 100 nM plasmin for 5 min had no effect on the peak amplitude, latency, or duration of the $[Ca^{2+}]_i$ response to $PAF_{16}$. Therefore, the effect of plasmin on the cellular response to thrombin is selective.

## DISCUSSION

Despite the diverse array of cellular responses to thrombin, $[Ca^{2+}]_i$ transients resulting from $Ca^{2+}$ influx and/or intracellular $Ca^{2+}$ mobilization represent a universal and rapid effect [28–33]. Therefore the $[Ca^{2+}]_i$ response was used to monitor the effects of plasmin on thrombin stimulation of C6 cells. The demonstration of a $[Ca^{2+}]_i$ transient in response to thrombin-agonist peptide and the lack of a response to PPACK-inactivated thrombin strongly suggest that the C6-cell thrombin receptor is equivalent, or very similar to, the cloned platelet receptor [1,3,5].

Thrombin-receptor activation is initiated after proteolytic cleavage of the $Arg^{41}$-$Ser^{42}$ peptide bond [34]. The new N-terminus then functions as a tethered peptide ligand, binding to a separate site in the receptor [26,35,36]. In this investigation, we demonstrated that plasmin modifies the ability of C6 cells to respond to thrombin. Since plasminogen and DIP-plasmin had no effect, the most likely mechanism whereby plasmin modifies the thrombin response is direct proteolytic modification of the thrombin receptor. One explanation for our results is that plasmin slowly cleaves the same peptide bond that thrombin cleaves ($Arg^{41}$-$Ser^{42}$), causing receptor inactivation/internalization [37,38]. Ishii et al. [37] proposed that the cellular response to thrombin is a linear function of the number of proteolytically cleaved thrombin receptors. As extremely high concentrations of plasmin failed to directly generate a $[Ca^{2+}]_i$ transient, it is not likely that plasmin exclusively cleaves the $Arg^{41}$-$Ser^{42}$ peptide bond. Other plasmin-sensitive bonds may be located anywhere within the extracellular domains of the receptor; however, the ligand-binding region represents a plausible target as the thrombin-receptor response to agonist peptide was also inhibited by plasmin.

Although the response of C6 cells to thrombin was significantly inhibited by pretreatment with plasmin, a partial response remained. When cells were pre-incubated with plasmin for more than 5 min, the response to thrombin or agonist peptide was not further reduced (results not shown). Furthermore, increasing the concentration of thrombin or agonist peptide did not overcome the inhibitory effect of plasmin pretreatment. Therefore, if plasmin causes complete inactivation or internalization of the thrombin receptor, then a sub-population of receptors is apparently plasmin resistant. Alternatively, our results could be explained if the plasmin-modified thrombin receptors are not internalized but are deficient in the coupling of ligand binding to intracellular responses [37,38].

The effects of plasmin on platelet responses to a variety of agonists including thrombin are complex and plasmin-concentration dependent [39,40]. The platelet inhibitory activity of plasmin may be the result of a generalized process: blocking the mobilization of arachidonic acid from membrane phospholipid pools [40]. The complete preservation of the PAF response in plasmin-treated C6 cells indicates that a generalized cellular inhibition mechanism is not operational here.

Prothrombin is expressed in multiple regions of the central nervous system [41]. Thrombin may also enter the central nervous system through a defective or incompletely formed blood–brain barrier. Neuronal cells respond to thrombin with neurite re-

traction and cell rounding [42–44]. Astrocytes lose stellation and proliferate [31,45]. In addition, thrombin-stimulated astrocytes secrete increased amounts of nerve growth factor-$\beta$ [46] and endothelin [47]. Therefore the selective effects of plasmin in modulating the astroglial response to thrombin may be physiologically significant during both development and brain injury. Thrombin and plasmin play opposite roles in haemostasis and the present studies suggest that an analogous relationship may exist in cell function.

This work was supported in part by grants T32 NS7236 and HL-45786 from the National Institutes of Health, U.S.A. J.E.H. is the recipient of Physician Scientist Award HL-02592. S.L.G. is the recipient of Research Career Development Award HL-02272.

## REFERENCES

1 Coughlin, S. R., Vu, T.-K. H., Hung, D. T. and Wheaton, V. I. (1992) J. Clin. Invest. **89**, 351–355
2 Fenton, J. W., II, Ofosu, F. A., Moon, D. G. and Maraganore, J. M. (1991) Blood Coagulation Fibrinolysis **2**, 69–75
3 Hung, D. T., Wong, Y. H., Vu, T.-K. H. and Coughlin, S. R. (1992) J. Biol. Chem. **267**, 20831–20834
4 Vu, T.-K. H., Hung, D. T., Wheaton, V. I. and Coughlin, S. R. (1991) Cell **64**, 1057–1068
5 Brass, L. F., Vassallo, R. R., Jr., Belmonte, E., Ahuja, M., Cichowski, I. and Hoxie, J. A. (1992) J. Biol. Chem. **267**, 13795–13798
6 Zhong, C., Hayzer, D. J., Corson, M. A. and Runge, M. S. (1992) J. Biol. Chem. **267**, 16975–16979
7 Plow, E. F., Felez, J. and Miles, L. A. (1991) Thromb. Hemostasis **66**, 32–36
8 Ponting, C. P., Marshall, J. M. and Cederholm-Williams, S. A. (1992) Blood Coagulation Fibrinolysis **3**, 605–614
9 Ellis, V. and Dano, K. (1992) Fibrinolysis **6** (Suppl. 4), 27–34
10 Hall, S. W., Humphries, J. E. and Gonias, S. L. (1991) J. Biol. Chem. **266**, 12329–12336
11 Shi, G.-Y., Hau, J.-S., Wang, S.-J., Wu, I.-S., Chang, B.-I., Lin, M. T., Chow, Y.-H., Chang, W.-C., Wing, L.-Y. C., Jen, C. J. and Wu, H.-L. (1992) J. Biol. Chem. **267**, 19363–19368
12 Snyder, F. (1990) Am. J. Physiol. **259**, C697–C708
13 Sturk, A., Cate, J. W. T., Hosford, D., Mencia-Huerta, J.-M. and Braquet, P. (1989) Adv. Lipid Res. **23**, 219–276
14 Hwang, S.-B. (1990) J. Lipid Mediators **2**, 123–158
15 Shukla, S. D. (1991) Lipids **26**, 1028–1033
16 Hanahan, D. J. (1992) Curr. Top. Cell. Regul. **33**, 65–78
17 Feuerstein, G., Tian-Li, Y. and Lysko, P. G. (1990) Stroke **21** (suppl. III), 90–94
18 Frerichs, K. U. and Feuerstein, G. Z. (1990) Cerebrovasc. Brain Metab. Rev. **2**, 148–160
19 Hall, S. W., Humphries, J. E. and Gonias, S. L. (1991) J. Biol. Chem. **266**, 12329–12336
20 Tas, P. W. L. and Koschel, K. (1990) Exp. Cell Res. **189**, 22–27
21 Kentroti, S., Baker, R., Lee, K., Bruce, C. and Vernadakis, A. (1991) J. Neurosci. Res. **28**, 497–506
22 Deutsch, D. G. and Mertz, E. T. (1970) Science **170**, 1095–1096
23 Chase, T. and Shaw, E. (1967) Biochem. Biophys. Res. Commun. **29**, 508–518
24 Anonick, P. K. and Gonias, S. L. (1991) Biochem. J. **275**, 53–59
25 Vassallo, R. R., Kieber-Emmons, T., Cichowski, K. and Brass, L. F. (1992) J. Biol. Chem. **267**, 6081–6085
26 Sabo, T., Gurwitz, D., Motola, L., Brodt, P., Barak, R. and Elhanaty, E. (1992) Biochem. Biophys. Res. Commun. **188**, 604–610
27 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
28 Tirruppathi, C., Lum, H., Andersen, T. T., Fenton, J. W., II and Malik, A. B. (1992) Am. J. Physiol. **263**, L595–L601
29 Jones, L. G., McDonough, P. M. and Brown, J. H. (1989) Mol. Pharmacol. **36**, 142–149
30 Davies, T. A., Drotts, D. L., Weil, G. J. and Simons, E. R. (1989) J. Biol. Chem. **264**, 19600–19606
31 Trejo, J., Chambard, J.-C., Karin, M. and Brown, J. H. (1992) Mol. Cell. Biol. **12**, 4742–4750
32 Kovács, T., Tordai, A., Szász, I., Sarkadi, B. and Gárdos, G. (1990) FEBS Lett. **266**, 171–174
33 Magócsi, M., Sarkadi, B., Kovács, T. and Gárdos, G. (1989) Biochim. Biophys. Acta **984**, 88–96
34 Vu, T.-K. H., Wheaton, V. I., Hung, D. T., Charo, I. and Coughlin, S. R. (1991) Nature (London) **353**, 674–677
35 Scarborough, R. M., Naughton, M., Teng, W., Hung, D. T., Rose, J., Vu, T.-K. H., Wheaton, V. I., Turck, C. W. and Coughlin, S. R. (1992) J. Biol. Chem. **267**, 13146–13149
36 Ni, F., Ripoll, D. R., Martin, P. D. and Edwards, B. F. P. (1992) Biochemistry **31**, 11551–11557
37 Ishii, K., Hein, L., Kobilka, B. and Coughlin, S. R. (1993) J. Biol. Chem. **268**, 9780–9786
38 Hoxie, J. A., Ahuja, M., Belmonte, E., Pizarro, S., Parton, R. and Brass, L. F. (1993) J. Biol. Chem. **268**, 13756–13763
39 Schafer, A. I., Maas, A. K., Ware, J. A., Johnson, P. C., Rittenhouse, S. E. and Salzman, E. W. (1986) J. Clin. Invest. **78**, 73–79
40 Schafer, A. I. and Adelman, B. (1985) J. Clin. Invest. **75**, 456–461
41 Dihanich, M., Kaser, M., Reinhard, E., Cunningam, D. and Monard, D. (1991) Neuron **6**, 575–581
42 Gurwitz, D. and Cunningham, D. D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3440–3444
43 Herrick-Davis, K., Camussi, G., Bussolino, F. and Balioni, C. (1991) J. Biol. Chem. **266**, 18620–18625
44 Jalink, K. and Moolenaar, W. H. (1992) J. Cell Biol. **118**, 411–419
45 Cavanaugh, K. P., Gurwitz, D., Cunningham, D. D. and Bradshaw, R. A. (1990) J. Neurochem. **54**, 1735–1743
46 Neveu, I., Frédéric, J., Jandrot-Perrus, M., Wion, D. and Brachet, P. (1993) J. Neurochem. **60**, 858–867
47 Ehrenreich, H., Costa, T., Clouse, K. A., Pluta, R. M., Ogino, Y., Coligan, J. E. and Burd, P. R. (1993) Brain Res. **600**, 201–207

Received 29 July 1993; accepted 16 August 1993