Regulation of neurite outgrowth from differentiated human neuroepithelial cells: a comparison of the activities of prothrombin and thrombin

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The mechanism by which thrombin and prothrombin control neurite retraction was studied in Ad12E1HER10 human neuroepithelial cells. Morphological changes in differentiated cells were apparent within minutes of the addition of very low concentrations of thrombin (3 pM). Higher concentrations (2 nM) of prothrombin were required to elicit a similar response. Doses of thrombin and prothrombin sufficient to cause neurite retraction stimulated protein tyrosine kinase activity. Protein tyrosine kinase activation also correlated positively with thrombin- and prothrombin-induced phosphoinositide 3-kinase activation and InsP_3 dephosphorylation. However, thrombin-stimulated $\text{Ins}(1,4,5)\text{P}_3$ generation and intracellular Ca^{2+} mobilization only occurred at concentrations in excess of those needed to induce retraction. No fluctuations in $\text{Ins}(1,4,5)\text{P}_3$ were detected after stimulation with prothrombin, and no rapid synchronized release of Ca^{2+} was observed, even at very high concentrations. Prothrombin did, however, cause small oscillations in the intra-

cellular Ca^{2+} concentration, similar to those produced by low concentrations of thrombin, after ~ 30 min. We conclude that prothrombin- and thrombin-induced neurite retractions are not dependent on $\text{PtdIns}(4,5)\text{P}_2$ and Ca^{2+} mobilization, but are more probably mediated through an effector mechanism involving protein tyrosine kinase activation. No intracellular Ca^{2+} mobilization, protein tyrosine kinase activity or neurite retraction was observed after treatment of cells with proteolytically inactive mutant thrombin (S205 → A). Prothrombin-mediated intracellular Ca^{2+} mobilization and neurite retraction were inhibited by hirudin, which was shown to interact with thrombin but not prothrombin. It is concluded that cleavage of prothrombin to thrombin is a necessary prerequisite for biological activity on differentiated Ad12E1HER10 cells and that differences in agonist concentration are capable of coupling the thrombin receptor to different pathways within the cell.

INTRODUCTION

Thrombin is a multi-functional serine proteinase that regulates the growth characteristics, morphology and biochemistry of a variety of mammalian cell types involved in haemostasis and wound healing. It can, for example, activate blood platelets [1,2], act as a mitogen for fibroblasts or lymphocytes [3,4] and macrophages [5], and evoke several responses in endothelial cells [6–8]. However, it also influences the behaviour of other cells, inhibiting neurite outgrowth from cultured neuronal cells [9–11] and stimulating bone resorption [12–14].

It is possible that all of these cellular responses to thrombin are mediated through the recently cloned thrombin receptor [15,16]. This molecule, a novel member of the ‘seven-span’ superfamily of G-protein-coupled receptors, comprises seven transmembrane domains, a C-terminal cytoplasmic tail and a long N-terminal extracellular region which contains a thrombin recognition site [15,16]. Binding of thrombin is thought to provoke a change in receptor conformation [17], followed by thrombin-catalysed cleavage to release a 41-residue N-terminal peptide. The new N-terminus then functions as a tethered agonistic ligand that activates the receptor, and thus initiates cellular responses to thrombin stimulation. This model of receptor activation is confirmed by the observation that peptides which mimic the new N-terminal sequence can duplicate most of the cellular effects of thrombin [18–21].

Considerable information is available on the biochemical consequences of thrombin receptor activation, but understanding

of the mechanisms by which the tethered N-terminal ligand stimulates the various responses of target cells remains incomplete. Receptor stimulation causes both increased activity of phosphoinositidase C and pertussis-toxin-sensitive inhibition of adenylate cyclase [22,23], with the 1,2-diacylglycerol and $\text{Ins}(1,4,5)\text{P}_3$ formed by phosphoinositidase C then stimulating protein kinase C and mobilizing intracellular Ca^{2+} stores [24]. Thrombin also stimulates tyrosine kinase activity [25–29], activates mitogen-activated protein (MAP) kinases [30] and increases GTP binding to Ras [31,32]. The inter-relationships between these responses are still not clear, but it is now apparent that stimulation of thrombin receptors does not always produce exactly the same pattern of biochemical response and does not always lead to increased DNA synthesis and mitogenesis.

This heterogeneity of responses is particularly evident in experiments that compare cellular responses to thrombin and to synthetic peptides that mimic the effects of the receptor-tethered ligand. For example, treatment of hamster fibroblasts with thrombin gives rise to a prolonged activation of MAP kinase, whereas the receptor peptide SFLLRNP produces only a transient increase in activity [30]. Moreover, a peptide ligand with a single amino acid substitution can act as a partial agonist that causes a shape change of human platelets, but provokes no Ca^{2+} mobilization, protein kinase C activation, secretion or aggregation [33]. It is not yet clear whether such observations require the existence of more than one thrombin receptor or can be explained in terms of different responses that are all mediated in some way through a single molecule.

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; dbcAMP, dibutyl cyclic AMP; DIP, di-isopropylfluorophosphate; FCS, fetal calf serum; PI3K, phosphoinositide 3-kinase; DMEM, Dulbecco's modified Eagle's medium; MAP kinase, mitogen-activated protein kinase.

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Outgrowth of neurites from cultured epithelial cells is modulated both by thrombin and by glia-derived nexin (GDN), a naturally occurring inhibitor [9,34–37]. It is presumed that regulation by thrombin comes into play when a trauma to the central nervous system compromises the blood–brain barrier and activates thrombin formation. Such trauma is, of course, not essential for the normal regulation of neuronal cell differentiation, and prothrombin, which is the proenzyme precursor of thrombin and an abundant plasma protein, also induces neurite retraction both in primary cultures of human neuroepithelial cells and in the transformed human cell line Ad12E1HER10 [38,39]. The likelihood that prothrombin is a biologically relevant regulator of neurite outgrowth is greatly enhanced by the fact that the responsive cells themselves express prothrombin mRNA [40]. Previous results have suggested that prothrombin's action requires its conversion to thrombin at the surface of responsive cells, followed by activation of the thrombin receptor [39].

We have now undertaken a much more extensive examination of the action of prothrombin on differentiated neuronal cells, in particular by comparing the abilities of thrombin and prothrombin to provoke several different responses [neurite retraction, protein tyrosine phosphorylation, phosphoinositide breakdown, phosphoinositide 3-kinase (PI3K) activation and intracellular Ca^{2+} mobilization]. The results confirm that for cells to respond to prothrombin it must be converted to thrombin, and they also indicate some notable quantitative differences in the relative sensitivities of different responses to these two stimuli.

MATERIALS AND METHODS

Cells

Ad12E1HER10 cells were produced by transfection of human embryo retinoblasts (HERs) with adenovirus early region 1 (E1) DNA [41]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 8% fetal calf serum (FCS) and 2 mM glutamine in plastic tissue culture dishes. Cells were differentiated by treatment with 2 mM dibutyryl cyclic AMP (dbcAMP) in supplemented serum-free DMEM [42]. Additional dbcAMP was added every second day to maintain the differentiated phenotype.

For neurite retraction assays, Ad12E1HER10 cells were plated at low density in 5 cm plastic tissue culture dishes and differentiated for 2 days. Reversal of differentiation was initiated by the addition of thrombin or prothrombin (both obtained from Calbiochem). The number of neuritic processes (with a length in excess of two cell bodies) was then counted directly (i.e. without fixing) for four sets of 50 cells at each time point.

Mutant thrombin (Ser to Ala at residue 205) which has no proteolytic activity was expressed in baby hamster kidney cells and purified by immunoaffinity chromatography using an antibody which recognizes human prothrombin.

Assays of thrombin activity

The proteolytic activities of the enzyme and proenzyme were assayed using a synthetic substrate (Channel Diagnostics) essentially as described previously [39].

Single-cell measurements of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

Cells were grown on coverslips, and incubated with 3 μM Indo-1-AM (Sigma) for 30–40 min at 37 °C in culture medium. The coverslips were then mounted in a perspex chamber and the cells perfused with Hank's Balanced Salt solution (Gibco) including

10 mM Hepes, pH 7.2, for 10–15 min to allow de-esterification of the Indo-1 AM before $[\text{Ca}^{2+}]_i$ measurements were made. Cells were monitored using an ultraviolet laser-scanning confocal imaging system (BioRad MRC-600). $[\text{Ca}^{2+}]_i$ values were calculated by using Ca^{2+} -saturated and Ca^{2+} -depleted Indo-1 solutions for R_{max} and R_{min} , respectively [43]. Ca^{2+} measurements were carried out at room temperature. For the concentration–response curve for thrombin-stimulated increases in $[\text{Ca}^{2+}]_i$, the area under the response curve was normalized to the maximum thrombin response, obtained with 10 nM thrombin. Data were fitted to the Hill equation to estimate the concentration of agonist causing a half-maximal response (EC_{50}).

Interaction of thrombin and prothrombin with hirudin

Recombinant hirudin (25 μg) (Calbiochem) was labelled with ^{125}I using the chloramine T protocol. Unbound ^{125}I was removed from the polypeptide by chromatography on Sephadex G25 eluted with 0.2 M Tris/HCl, pH 7.5, containing 1% (w/v) BSA. Fractions containing radiolabelled hirudin were collected and stored at –20 °C until required.

For the binding experiments ^{125}I -labelled hirudin (2.5×10^6 c.p.m.) was incubated alone or with thrombin or prothrombin for 1 h in 10 mM Tris/HCl, pH 7.2, 0.15 M NaCl, 1% Nonidet P40 (buffer A). Samples were pre-cleared by the addition of Sepharose–Protein G (Sigma) for 30 min. After centrifugation each supernatant was divided in two; one aliquot was incubated with a sheep polyclonal antibody raised against human prothrombin (10 μl) (Biogenesis) for 30 min, while the other was incubated with no addition. Sepharose–Protein G was again added to each tube. After a further 15 min, samples were centrifuged and the pellet washed three times with buffer A. Immunoprecipitated proteins were fractionated by SDS/PAGE and radiolabelled hirudin was detected by autoradiography of dried gels.

Analysis of the inositol phosphates of Ad12E1HER10 cells

Cells were grown in DMEM supplemented with 8% dialysed FCS in the presence of 2 $\mu\text{Ci}/\text{ml}$ [^3H]inositol for 72 h, and then incubated in serum-free DMEM containing 2 $\mu\text{Ci}/\text{ml}$ [^3H]inositol and 2 mM dbcAMP for an additional 72 h. The labelled cells were incubated in serum-free DMEM prior to addition of stimuli. Incubations were terminated by adding 750 μl of 8% (v/v) perchloric acid (containing 10 mM EDTA and 0.5 mg/ml phytic acid hydrolysate) to rinsed cells. After 20 min on ice, supernatant and cell debris were removed from the dish by aspiration and centrifuged. The resulting extract was neutralized using 1.2 M KOH, 10 mM Hepes and 10 mM EDTA. Neutralized [^3H]inositol-labelled samples were analysed by anion-exchange chromatography using a Whatman Partisphere 5 μm SAX HPLC column eluted with a gradient of ammonium dihydrogen orthophosphate, pH 3.8 [44]. Radioactivity in the eluate was monitored, and ^3H -labelled inositol phosphates were identified by comparison of their elution characteristics with those of ^3H -labelled standards (Amersham).

PI3K assays

Cells stimulated with thrombin or prothrombin were lysed in ice-cold lysis buffer (137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl_2 , 1.0 mM CaCl_2 , 0.5 mM Na_2VO_3 , 1% Nonidet P-40, 10% glycerol, 20 mM Tris/HCl, pH 8.0, 10 $\mu\text{l}/\text{ml}$ leupeptin and 2 mM PMSF). p85 α was immunoprecipitated from cell lysates, using monoclonal antibody (mAb) U13 coupled to Protein A–Sepharose [45]. Immunoprecipitates were assayed

directly for PI3K activity using [γ - 32 P]ATP and PtdIns substrate, as described by Jackson et al. [46]. 32 P-labelled lipids were separated on oxalate-impregnated Whatman LK6D TLC plates using chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.). [32 P]PtdIns3P was scraped from the plate and quantified by liquid scintillation counting.

Protein tyrosine phosphorylation in Ad12E1HER10 cells

Cells were grown in 5 cm plastic tissue culture dishes and differentiated by the addition of dbcAMP in serum-free defined medium on day 1 and day 3. Experiments were performed on day 4. Cells were treated with thrombin, mutant thrombin, prothrombin or FCS for the appropriate times. Reactions were stopped by removing the medium containing the stimulating molecule followed by briefly rinsing with ice-cold saline containing 0.4 mM sodium vanadate, 10 mM NaF and 10 mM sodium pyrophosphate. A 300 μ l aliquot of 9 M urea containing 50 mM Tris/HCl, pH 7.4, 0.15 M β -mercaptoethanol, 0.4 mM sodium vanadate and 10 mM NaF was added, and the samples were sonicated and stored at -20°C . Aliquots (50 μ g of protein) were fractionated on polyacrylamide gels (12%) and then transferred to nitrocellulose filters which were incubated with an anti-phosphotyrosine monoclonal antibody (UBI). Tyrosine-phosphorylated proteins were visualized using the ECL protocol (Amersham). Di-isopropylfluorophosphate-modified thrombin (DIP-thrombin) and DIP-prothrombin were prepared by the method described previously [38].

RESULTS

Response of differentiated Ad12E1HER10 cells to thrombin and prothrombin

It has previously been shown that Ad12E1HER10 cells serve as an excellent model for the differentiation of human neuroepithelial cells [38,39,42]. Addition of dbcAMP, in the absence of FCS, produces a differentiated phenotype exemplified by 'rounded up' cell bodies and the extension of neuritic-type processes (compare Figures 1a and 1b). Differentiation can be reversed by FCS, thrombin or prothrombin [38]. To assess the relative abilities of the latter two proteins to produce a phenotypic change, Ad12E1HER10 cells were differentiated for 2 days and the proportion of cells in which reversal of differentiation occurred was measured (as neurite retraction at various times after addition of the agonists). Examples of agonist-treated cultures are presented in Figure 1, and the time courses of neurite retraction in response to different doses of prothrombin and thrombin are shown in Figure 2. Thrombin was considerably more potent in this assay, higher concentrations of prothrombin being required to return cells to the undifferentiated phenotype. A comparison of Figures 1c, 1d and 1e shows that the morphology of the cell bodies changed rapidly, becoming much flatter and more fibroblastic in appearance. By 30 min (Figures 1d and 1f) most of the neuritic processes had retracted. Comparison of Figures 1c and 1e (10 min after addition of agonist) with Figures 1d and 1f (after 30 min) shows the greater potency of thrombin. Neurite retraction occurred in about half of the responsive cells after treatment with 2 nM prothrombin for $\sim$ 30 min, whereas 3 pM thrombin produced a similar response in half that time (Figure 2). Prothrombin at < 1 nM had relatively little effect. The observed responses to prothrombin tended to vary between experiments rather more than with thrombin, giving rise to artefactual plateaus seen in some of the curves in Figure 2(b). However, the overall sensitivities of neurite retraction to prothrombin were reproducible in different experiments. While the

Figure 1 Differentiation of Ad12E1HER10 cells and their response to thrombin and prothrombin

(a) Cells grown in serum-supplemented medium; (b) cells differentiated by the addition of 2 mM dbcAMP in serum-free medium for 24 h; (c) as (b) but 10 min after the addition of 10 nM prothrombin; (d) as (c) but 30 min after addition of prothrombin; (e) as (b) but 10 min after addition of 1 nM thrombin; (f) as (e) but 30 min after addition of thrombin.

majority of differentiated cells responded rapidly to both agonists (when in excess of their minimal concentrations; Figure 2) a proportion appeared to be unaffected (Figure 2). Addition of proteolytically inactive mutant thrombin at a concentration of 7 nM produced no morphological response in differentiated cells up to 24 h.

Effects of thrombin and prothrombin on $[\text{Ca}^{2+}]_i$ in Ad12E1HER10 cells

Thrombin causes a rapid rise in $[\text{Ca}^{2+}]_i$ in several cell types [14,47–49]. In order to establish whether prothrombin elicits similar responses, changes in $[\text{Ca}^{2+}]_i$ were monitored following addition of both stimuli.

Figure 3 shows the effects on $[\text{Ca}^{2+}]_i$ of introducing thrombin and prothrombin into the cell perfusion system. Thrombin caused a rapid increase in $[\text{Ca}^{2+}]_i$ which returned to basal levels within 1–2 min. This increase was mainly fuelled by Ca^{2+} release from intracellular stores (results not shown) and was dependent on the thrombin concentration (Figure 3, inset). Analysis of these data

Figure 2 Neurite retraction of differentiated Ad12E1HER10 cells in response to thrombin and prothrombin

Ad12E1HER10 cells were differentiated by the addition of 2 mM dbcAMP for 2 days. Various concentrations of thrombin (a) or prothrombin (b) were added and the number of cells with neurites (with length greater than twice the size of the cell body) was counted at the times indicated (see the Materials and methods section). Four sets of fifty cells each were counted for each time point, the value plotted being the mean of these. Thrombin concentrations: □, control; ■, 0.675 pM; ●, 3.3 pM; ○, 33.7 pM; ▲, 337 pM; △, 3370 pM. Prothrombin concentrations: □, control; ○, 1.7 nM; ■, 2.4 nM; ●, 3.5 nM; △, 6.9 nM; ▲, 10.4 nM.

Figure 3 Effect of thrombin and prothrombin on mobilization of Ca^{2+} in single differentiated Ad12E1HER10 cells

Indo-1-loaded cells were exposed to either 10 nM thrombin (□) or 10 nM prothrombin (▼) (at arrow) included in the perfusion medium. To facilitate comparison of the two traces, the baseline of the cells exposed to prothrombin has been offset. Traces are representative of over 10 experiments, with 8–12 cells in each experiment. Inset: concentration–response curve for thrombin-stimulated $[Ca^{2+}]_i$ increases in individual HER 10 cells. The values represent increases in the area under the curve above resting levels, normalized to the maximum response (10 nM thrombin).

according to the Hill equation yielded an estimate for the concentration of thrombin producing a half-maximal effect (EC_{50}) of 4.8 ± 0.2 nM (mean $\pm$ S.E.M.). Comparison of these data with the results of the neurite retraction assays (Figure 2) suggests that the changes in cellular morphology were induced by

concentrations of thrombin much lower than those required to induce rapid intracellular Ca^{2+} release.

In contrast to the results obtained with thrombin, no immediate changes in $[Ca^{2+}]_i$ were seen in response to prothrombin (concentrations up to 150 nM). However, after 25–45 min, cells started to show $[Ca^{2+}]_i$ oscillations in response to 10 nM prothrombin (Figure 4a). Cells incubated in medium alone showed no such activity. The $[Ca^{2+}]_i$ oscillations in individual cells were not synchronized, and they varied in frequency and amplitude, with a small percentage of cells not responding at all. Exposure to thrombin after prothrombin-induced $[Ca^{2+}]_i$ oscillations produced a reduced response compared with that seen with thrombin alone (Figure 3 and results not shown), indicating the depletion of a shared Ca^{2+} pool (Figure 4a). To investigate the genesis of the prothrombin-induced $[Ca^{2+}]_i$ oscillations, the thrombin antagonist hirudin was used. The rise in $[Ca^{2+}]_i$ provoked by a submaximal concentration of thrombin (0.25 nM) was blocked by the addition of hirudin (0.28 unit/ml). The $[Ca^{2+}]_i$ oscillations induced by 10 nM prothrombin were also attenuated by similar concentrations of hirudin, added directly to the perfusion chamber once the oscillations had started (results not shown).

Treatment of Ad12E1HER10 cells with submaximal concentrations of thrombin (e.g. 0.25 nM), produced $[Ca^{2+}]_i$ oscillations comparable with those evoked by prothrombin, but without any appreciable delay. These oscillations continued for > 30 min (Figure 4b). The concentration of Ca^{2+} released, however, was appreciably lower than that seen after the addition of large doses of thrombin. At concentrations of thrombin below 0.1 nM the oscillations were not observed even at later times (up to 1 h). The addition of proteolytically inactive mutant thrombin (10 nM) produced no rapid initial increase in $[Ca^{2+}]_i$ nor $[Ca^{2+}]_i$ oscillations up to 1 h.

Changes in inositol phosphate levels in stimulated cells

In view of the differences between intracellular Ca^{2+} changes provoked by thrombin and prothrombin, investigations were carried out to compare the levels of inositol phosphates in the

Figure 4 Calcium oscillations in single differentiated Ad12E1HER10 cells evoked by prothrombin and thrombin

(a) Cells were perfused with 10 nM prothrombin (first arrow) and the perfusion was then stopped. The response to thrombin was tested by perfusion of 10 nM thrombin (second arrow). (b) Cells were perfused with a submaximal concentration of thrombin (0.25 nM) (arrow). Traces are representative of four experiments, with 8–12 cells in each experiment.

cells after stimulation by the two proteins. Treatment of cells with thrombin (10 nM) causes rapid formation of $\text{Ins}(1,4,5)\text{P}_3$ and its immediate metabolites $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ [50] within 1 min (Figure 5). By contrast, no changes were detected during stimulation with prothrombin (10 nM) (Figure 5). The data shown (Figure 5b) were obtained after stimulation for 1 min, but identical elution patterns were seen at 0.15, 0.5, 5, 10, 30 and 45 min after addition of prothrombin (results not shown). Even in the presence of 20 mM LiCl no changes from control, unstimulated cells could be observed.

It seems likely that the absence of detectable $\text{Ins}(1,4,5)\text{P}_3$ formation in response to prothrombin reflects the inability of our assays to detect the formation of the small concentrations of $\text{Ins}(1,4,5)\text{P}_3$ needed to initiate Ca^{2+} oscillations, rather than the complete absence of $\text{Ins}(1,4,5)\text{P}_3$. It has previously been suggested that the action of $\text{Ins}(1,4,5)\text{P}_3$ is highly co-operative and that very low concentrations are needed to open a few Ca^{2+} channels [51].

Both thrombin (10 nM) and prothrombin (10 nM) caused a rapid but transient accumulation of the predominant InsP_5 isomer (Figure 6), which was likely to be $\text{Ins}(1,3,4,5,6)\text{P}_5$ (see

Figure 5 Thrombin- and prothrombin-induced changes in inositol phosphates in differentiated Ad12E1HER10 cells

Differentiated Ad12E1HER10 cells were labelled to equilibrium with $[\text{H}]$ inositol as described in the Materials and methods section and stimulated with either thrombin or prothrombin. $[\text{H}]$ inositol phosphates were separated by HPLC. (a) Control unstimulated cells; (b) cells stimulated for 1 min with 10 nM prothrombin; (c) cells stimulated for 1 min with 10 nM thrombin. Inositol phosphate peaks are identified in profile (c) by comparison with standards.

[52]), and a reciprocal decline in InsP_5 . Reversion to pre-stimulated levels took approximately 30 min following stimulation with thrombin and prothrombin.

PI3K activity in thrombin- and prothrombin-stimulated cells

In human platelets, activation by thrombin provokes rapid synthesis of $\text{PtdIns}(3,4,5)\text{P}_3$ and a more gradual increase in the levels of $\text{PtdIns}(3,4)\text{P}_2$, consistent with the action of a PI3K directed against $\text{PtdIns}(4,5)\text{P}_2$ [53]. We examined the regulation of a PI3K activity in cells responding to thrombin and prothrombin by performing *in vitro* kinase assays on $\text{p85}\alpha$ immunoprecipitates. Both thrombin (10 nM) and prothrombin (10 nM) increased PI3K activity, as shown by enhanced synthesis of $[\text{P}]$ $\text{PtdIns}3\text{P}$ from PtdIns and $[\text{P}]$ ATP (Figure 7). There was an approximately 2-fold increase over basal activity after stimulation for 1 min, and the activity remained elevated 5 min after stimulation but had returned to pre-stimulated levels after 30 min.

Protein tyrosine phosphorylation in response to prothrombin and thrombin

It is now well established that cellular proteins are rapidly phosphorylated on tyrosine residues after thrombin stimulation.

Figure 6 Thrombin- and prothrombin-induced changes in InsP_6 and InsP_5 levels in differentiated Ad12E1HER10 cells

Differentiated cells were labelled with [^3H]inositol as described in the Materials and methods section, stimulated with agonist for the times indicated and then processed as described. Inositol phosphates were separated by HPLC. Relative concentrations of InsP_6 (○) and InsP_5 (●) at various times after 10 nM thrombin (a) and 10 nM prothrombin (b) stimulation are shown.

Figure 7 Thrombin- and prothrombin-induced changes in PI3K activity in differentiated Ad12E1HER10 cells

Differentiated Ad12E1HER10 cells were stimulated with either 10 nM thrombin (Thr) or 10 nM prothrombin (Pro), harvested after the allotted times and then subjected to immunoprecipitation using the monoclonal antibody U13, directed against the p85 α subunit of PI3K. After incubation with PtdIns and Mg^{2+} ATP, lipids were separated on Whatman LK6D TLC plates. Radioactive spots were detected by autoradiography, eluted from the plate and radioactivity was counted.

We therefore compared the relative activities of prothrombin and thrombin in stimulating protein tyrosine phosphorylation. Phosphorylation was detected by Western blotting of cell extracts using an antibody which recognized tyrosine phosphate residues. After addition of thrombin (25 pM), there was rapid

phosphorylation of three major proteins of molecular mass 62, 100 and 200 kDa (Figure 8a); the phosphorylation of other, less prominent, bands became apparent after prolonged stimulation. Addition of prothrombin at concentrations (5 nM) which caused rapid neurite retraction but no initial Ca^{2+} mobilization produced a pattern of protein phosphorylation similar to that seen with thrombin (Figure 8b), although modification of some bands became apparent at later times: for example, phosphorylation of the 62 kDa protein was seen after 2 min with thrombin but only after 25 min with prothrombin. The most prominent bands seen after Western blotting were similar to those reported previously [24,28]. In addition to the bands that were phosphorylated in response to stimulation, a 60 kDa protein already phosphorylated in differentiated Ad12E1HER10 cells (marked with an arrow in Figures 8a and 8b) rapidly became dephosphorylated in response to thrombin and prothrombin.

Modification of thrombin with DIP blocks its active site and abolishes its proteolytic and mitogenic activity, whereas incubation of prothrombin with DIP produces no change in its ability to cause neurite retraction [38]. Addition of DIP-modified proteins to differentiated cells produced effects consistent with there being a requirement for prothrombin to be converted to thrombin in order to stimulate tyrosine phosphorylation: DIP-thrombin caused little increase in protein phosphorylation, whereas there was virtually no difference between DIP-treated prothrombin and the native protein (Figures 8a and 8b).

Similarly, the addition of proteolytically inactive mutant thrombin (7 nM) caused no change in the pattern of protein tyrosine phosphorylation (results not shown). Addition of genistein (20 μM) to differentiated Ad12E1HER10 cells prior to stimulation with thrombin or prothrombin reduced but did not abolish tyrosine phosphorylation, and also reduced the ability of thrombin and prothrombin to induce neurite retraction (results not shown).

Cell-mediated conversion of prothrombin to thrombin

Previous studies have suggested that small amounts of free thrombin may be formed during treatment of differentiated Ad12E1HER10 cells with prothrombin [39]. This phenomenon has been examined using a chromogenic proteinase assay. Incubation of prothrombin with Ad12E1HER10 cells at 37 °C for 1 h in DMEM resulted in conversion of at least a fraction of the prothrombin to thrombin (Figure 9). If, however, incubations were carried out in PBS (results not shown) or in medium containing EDTA (Figure 9) the prothrombin remained inactive; addition of Ca^{2+} and Mg^{2+} restored the proteolytic activity (Figure 9). Addition of prothrombin to differentiated cells in PBS rather than in DMEM did not result in neurite retraction. We conclude, therefore, that bivalent cations are essential for the cell-mediated conversion of prothrombin to thrombin, and that this conversion is necessary for the prothrombin-mediated morphological changes associated with neurite retraction.

Interaction of hirudin with thrombin and prothrombin

To confirm that the attenuation of the mobilization of intracellular Ca^{2+} after addition of hirudin to prothrombin was due to complex formation between the polypeptide and newly formed thrombin rather than direct binding to the proenzyme, the interaction of hirudin with the two proteins was compared. It can be seen from the autoradiograph presented in Figure 10 that when a mixture of prothrombin and ^{125}I -hirudin was immunoprecipitated with an antibody which recognized pro-

Figure 8 Tyrosine kinase activity in thrombin- and prothrombin-stimulated differentiated Ad12E1HER10 cells

Differentiated cells were treated with agonist for the times indicated, harvested and then subjected to PAGE and Western blotting using an antibody which recognizes tyrosine phosphate residues. (a) Cells stimulated with thrombin (Thr) (25 pM) or DIP-treated thrombin (DIP-Thr) as indicated. The period of stimulation is shown under the appropriate track; C, control unstimulated cells. An overexposure of the central portion of the autoradiograph is shown underneath. A protein band which is rapidly dephosphorylated upon agonist stimulation is indicated by >. (b) Cells stimulated with prothrombin (Pro) (5 nM) or DIP-treated prothrombin (DIP-Pro); C, control unstimulated cells. (c) Differentiated cells stimulated with thrombin (Thr) (25 pM) or with thrombin after treatment with genistein (Gen). (d) Differentiated cells stimulated with prothrombin (Pro) (5 nM) or with prothrombin after treatment with genistein (Gen); C, control undifferentiated cells. The positions of molecular mass markers are indicated. o, origin of the gel.

Figure 9 Proteolytic activity of prothrombin after incubation with differentiated Ad12E1HER10 cells

Prothrombin (14 pmol) was incubated with differentiated Ad12E1HER10 cells for 1 h in DMEM. 1, 5×10^4 cells; 2, 10^5 cells; 3, 10^6 cells; 4, 5×10^6 cells; 5, 10^6 cells in the presence of 5 mM EDTA; 6, no cells; 7, 10^6 cells in PBS + 0.5 mM CaCl_2 and 0.5 mM MgCl_2 . The proteolytic activity of the protein was then determined using the chromogenic substrate which is cleaved by thrombin (see the Materials and methods section and [39]). This was compared with known amounts of thrombin included in the assay. Bars: 8, 1.4 pmol; 9, 2.7 pmol; 10, 5.4 pmol; 11, 8 pmol.

thrombin, no radioactivity could be detected after SDS/PAGE (track 6). However, immunoprecipitation and electrophoresis of a thrombin/ ^{125}I -hirudin mixture demonstrated complex formation (track 7).

Figure 10 Interaction of hirudin with thrombin and prothrombin

^{125}I -labelled hirudin was incubated with human thrombin or prothrombin and then subjected to immunoprecipitation using a sheep polyclonal antibody against prothrombin (and thrombin). Precipitated proteins were fractionated by SDS/PAGE and co-precipitated ^{125}I -hirudin was detected by autoradiography. Track 1, purified ^{125}I -labelled hirudin; 2, ^{125}I -hirudin subjected to the immunoprecipitation protocol but with no antibody added; 3, as 2 but with prothrombin mixed with ^{125}I -hirudin; 4, as 2 but with thrombin mixed with ^{125}I -hirudin; 5, 6 and 7, as 2, 3 and 4 respectively but with antibody included in the immunoprecipitation reaction. The mobility of molecular mass markers is indicated.

DISCUSSION

It has been established previously that prothrombin is the factor present in FCS which is primarily responsible for the induction of neurite retraction in differentiated, transformed (Ad12E1HER10) and primary neuronal cells in culture [38,39].

Using purified reagents we have now shown that appreciably higher concentrations of prothrombin than thrombin are required to produce a similar morphological response [38,39; Figures 1 and 2). It has already been demonstrated that this activity is attributable to the protein itself and is not due to initial contamination by thrombin [38,39]. However, it has always seemed likely that cleavage of prothrombin would be required before the protein could become biologically active. Indeed, preliminary results have tended to suggest this, as the biological activity of prothrombin can be inhibited by glia-derived nexin, a specific inhibitor of thrombin [39]. In view of these data, it might be expected that prothrombin would elicit similar biochemical responses to those seen with thrombin, although perhaps slightly more slowly. The results presented in the present paper, however, show that this view is an over-simplification, and there appear to be distinct differences between the two agonists.

The data presented here confirm previous observations [22,23,30] that stimulation of the thrombin receptor in neuroepithelial cells by thrombin (Figure 3) or by a receptor peptide homologue (results not shown) provokes rapid formation of $\text{Ins}(1,4,5)\text{P}_3$ and mobilization of intracellular Ca^{2+} stores (Figures 3 and 5). Although changes in cellular morphology can be observed a few minutes after addition of prothrombin, we could find no evidence for a rapid synchronized release of calcium similar to that seen with thrombin (Figure 3). However, subsequent incubation led to the gradual appearance in some cells of $[\text{Ca}^{2+}]_i$ oscillations of a similar pattern to those seen with submaximal concentrations of thrombin (Figures 4a and 4b). This suggests that the prothrombin-induced $[\text{Ca}^{2+}]_i$ oscillations are probably caused by slow proteolysis of prothrombin releasing small amounts of thrombin. Studies with the thrombin antagonist hirudin confirm this, since it attenuated the prothrombin-induced $[\text{Ca}^{2+}]_i$ oscillations. However, no increase in $\text{Ins}(1,4,5)\text{P}_3$ was detectable either immediately following prothrombin addition or subsequently (Figure 5), probably because the very small rise in $\text{Ins}(1,4,5)\text{P}_3$ concentration required to trigger $[\text{Ca}^{2+}]_i$ oscillations in a limited number of cells would pass undetected using the currently available methods. It is important, however, to note that both agonists were capable of inducing morphological changes at concentrations far lower than those required for the release of Ca^{2+} .

Since no agonist-generated $\text{Ins}(1,4,5)\text{P}_3$ was observed, and it is likely that the response to thrombin has a similar mechanism, the elevation of $\text{Ins}(1,3,4,5,6)\text{P}_5$ after stimulation with prothrombin presumably arose from InsP_6 dephosphorylation. InsP_6 has been shown *in vitro* to be dephosphorylated to a number of InsP_5 isomers [including $\text{Ins}(1,3,4,5,6)\text{P}_5$] by an inositol polyphosphate phosphatase that was originally detected as $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase [54]. Activation of this enzyme has previously been reported during thrombin stimulation of human platelets [55].

Stimulation of the production of $\text{PtdIns}(3,4,5)\text{P}_3$ by a $\text{PtdIns}(4,5)\text{P}_2$ -directed PI3K activity is provoked both by receptor tyrosine kinases and by some agonists, including thrombin, which utilize heterotrimeric G-proteins to couple surface stimulation to phosphoinositidase C- β -directed $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis (see, for example, Stephens et al. [58]). Both thrombin and prothrombin produced a rapid stimulation of PI3K activity, but it is not clear at present whether this kinase activation is mediated by tyrosine phosphorylation or through a direct G-protein-dependent mechanism, nor how significant this activation is for the morphological changes associated with reversal of differentiation. The observation that prothrombin is able to induce changes in InsP_5 and InsP_6 metabolism and PI3K activity suggests that these processes would be sensitive to very low concentrations of thrombin, although it has not been definitively

demonstrated that they occur at agonist concentrations lower than those required for calcium mobilization but sufficient to induce neurite retraction. Additionally, it has not been shown unequivocally that InsP_6 dephosphorylation and PI3K activation are the same at very low concentrations of prothrombin and thrombin, although we have no reason to believe that this should not be so.

There is no doubt that thrombin and prothrombin both cause rapid increases in tyrosine phosphorylation of a similar set of polypeptides (Figure 8), consistent with the idea that both stimuli employ a similar (or the same) receptor. Additionally, the speed of this tyrosine phosphorylation is such that it could be involved in the regulation of neurite retraction (caused by thrombin and prothrombin). Similarly, the concentrations of agonists required to induce both neurite retraction and protein tyrosine phosphorylation are comparable, both processes being triggered by 25 pM thrombin and 2 nM prothrombin, at which concentrations no Ca^{2+} mobilization occurred.

The observation that prothrombin-induced neurite retraction and Ca^{2+} mobilization were inhibited by hirudin strongly suggests that the conversion of prothrombin to thrombin is essential for its biological activity when added to Ad12E1HER10 cells. The co-immunoprecipitation data presented in Figure 10 confirmed that hirudin failed to bind to prothrombin and was therefore very unlikely to inhibit an activity which was not reliant on cleavage to thrombin. Similarly, prothrombin was found to exhibit no activity in neurite-retraction assays in the presence of EDTA, another condition under which cell-mediated conversion to thrombin is inhibited (Figure 9). These data are consistent with our previous report that glia-derived nexin could inhibit neurite retraction [39], and very recent evidence which suggests that a novel proteinase capable of activating prothrombin exists on the surface of certain cell types [60]. Additional, although indirect, evidence that proteolytic activity derived from prothrombin is essential for its biological activity is provided by our demonstrations that proteolytically inactive mutant thrombin was unable to cause neurite retraction, Ca^{2+} mobilization or protein tyrosine phosphorylation. The obvious conclusion from these results is that simple binding to the cell surface by thrombin is insufficient to cause stimulation, and therefore that cleavage of the thrombin receptor is essential. By implication, binding of prothrombin to the cell surface is unlikely to trigger the observed responses. These conclusions are consistent with the observations that the thrombin receptor activating peptide (TRAP-508) [61] has no activity when added to differentiated Ad12E1HER10 cells (T. R. V. Pagliuca and R. J. A. Grand, unpublished work).

Previous studies have implied that not all of the observed cellular responses to thrombin are mediated through the cloned receptor [22,23,30]. However, the data presented have clearly demonstrated that different agonist concentrations can give rise to alternative intracellular responses. Thus, very low levels of thrombin added or generated from prothrombin by cells in the presence of bivalent cations are sufficient to activate protein tyrosine kinases and induce neurite retraction, and possibly to generate InsP_5 and activate PI3K. At higher concentrations of either thrombin or prothrombin Ca^{2+} is mobilized, probably following phosphoinositidase C activation, InsP_5 is generated and PI3K is activated. At very high concentrations of thrombin (which are never generated by cellular conversion of prothrombin) phosphoinositidase C is activated to such an extent that sufficient $\text{Ins}(1,4,5)\text{P}_3$ is produced to cause a rapid synchronized release of intracellular Ca^{2+} . This hypothesis is supported by recent evidence which demonstrates that differences in extracellular thrombin concentrations produce different rates of receptor activation which then govern the cells' response [62]

Additionally, recent evidence derived from a study of the lysophosphatidic acid receptor has confirmed that the extent of receptor occupancy can determine whether tyrosine kinases and/or heterotrimeric G-proteins are activated by a ligand [63].

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