Thrombin Receptor Activation Causes Rapid Neural Cell Rounding and Neurite Retraction Independent of Classic Second Messengers

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Abstract. The protease thrombin is a potent activator of various cell types. Thrombin cleaves and thereby activates its own seven-transmembrane-domain receptor which couples to G proteins. Thrombin also can inhibit neuronal differentiation, supposedly by degrading components of the extracellular matrix. Here we report that active thrombin induces immediate cell rounding and neurite retraction in differentiating N1E-115 and NG108-15 neural cells in serum-free culture. Serum (0.5–5% vol/vol) evokes similar responses, but the cell-rounding and neurite-retracting activity of serum is not attributable to thrombin. Neural cell rounding is transient, subsiding after 10–15 min, and subject to homologous desensitization, whereas retracted neurites rapidly degenerate. Thrombin action is inhibited by cytochalasin, but not colchicine. A novel 14-amino acid peptide agonist of the thrombin receptor fully mimics thrombin's morphoregulatory activity, indicating that thrombin-induced shape changes are receptor-mediated and not secondary to extracellular matrix degradation. Although thrombin receptors couple to phosphoinositide hydrolysis and Ca^{2+} mobilization, thrombin-induced shape changes appear to depend neither on the Ca^{2+}/protein kinase C- nor the cyclic nucleotide-mediated signal transduction pathways; however, the morphological response to thrombin is blocked by pervanadate, an inhibitor of tyrosine phosphatases, and by broad-specificity kinase inhibitors. Our results suggest that the thrombin receptor communicates to an as-yet-uncharacterized effector to reorganize the actin cytoskeleton and to reverse the differentiated phenotype of neural cells.

In addition to having a key role in the coagulation cascade, the protease thrombin is a potent activator of platelets and endothelial cells, and a mitogen for cells of mesodermal origin (for example see Shuman, 1986; Carney and Cunningham, 1978; Chambard et al., 1987). Thrombin activates its own G protein–coupled receptor, which spans the membrane seven times, through a unique proteolytic cleavage mechanism (Vu et al., 1991). Classic G protein–mediated signaling pathways in thrombin action include stimulation of phosphoinositide-specific phospholipase C, release of arachidonic acid, and pertussis toxin-sensitive inhibition of adenylate cyclase (reviewed by Siess, 1989). Thrombin receptors also are expressed in brain (Rasmussen et al., 1991) and are present on cultured neuronal cells (Snider et al., 1984, 1986; Snider, 1986; Means and Anderson 1986), while prothrombin is synthesized throughout the brain as well as in neural cell lines (Dihanich et al., 1991), suggesting that thrombin may serve a neurotransmitter or neuromodulatory function. On the other hand, thrombin can inhibit morphological differentiation of primary neuronal cells, astroglial, neuroepithelial and neuroblastoma cells (Hawkins and Seeds, 1986; Gurwitz and Cunningham, 1988; Monard, 1988; Grand et al., 1989; Nelson and Siman, 1990). The mechanism by which thrombin inhibits neurite outgrowth remains unknown but is believed to involve proteolytic degradation of components of the extracellular matrix (ECM)[1] (reviewed by Monard, 1988). This hypothesis seems plausible since thrombin can cleave ECM glycoproteins such as laminin and fibronectin (Liotta et al., 1981; Keski-Oja et al., 1981; Galdal et al., 1985), molecules that are known for their ability to promote neurite development. However, the possibility that thrombin modulates neuronal morphology by activating its own plasma membrane receptor, rather than degrading ECM components, has received relatively little attention.

In the present study, we have investigated the morphoregulatory activity of thrombin in murine NG108-15 and N1E-115 neural cells. These cell lines are widely used for studying various aspects of nerve cell function, including the regulation of differentiation (for review see Nirenberg et al., 1983; Hamprecht et al., 1985). Furthermore, N1E-115 cells constitute a convenient system to investigate early signaling events in the proliferative response (Moolenaar et al., 1979, 1981, 1984). In serum-containing medium, these cells express the phenotype of undifferentiated neuroblast-like cells, but they can be induced to extend neurites and to differentiate biochemically by serum removal. Both N1E-115 and NG108-15 cells express functional receptors and/or specific binding sites for numerous agonists, including thrombin

1. *Abbreviation used in this paper*: ECM, extracellular matrix.

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(∼30,000 high-affinity thrombin binding sites per NlE-115 cell [Snider, 1986; Snider et al., 1986]). Here we show that thrombin induces dramatic cell rounding and neurite retraction in a time-dependent manner, with an onset within 10–15 s of thrombin addition. Using a newly discovered peptide agonist which duplicates the NH_2-terminal "tethered" ligand of the receptor (Vu et al., 1991), we demonstrate that the rapid morphological response to thrombin is a receptor-mediated process and not secondary to proteolytic degradation of the ECM. In addition, we provide evidence that thrombin bypasses known G protein-dependent signal transduction pathways to elicit its morphological effects. Therefore, we have to postulate that the thrombin receptor communicates to an as-yet-unidentified effector to reorganize the cytoskeleton and to reverse morphological differentiation of neural cells.

Materials and Methods

Materials

Bovine and human thrombin, hirudin, dioctanoylglycerol, free fatty acids, prostaglandins, bradykinin and other neurotransmitters, colchicine, cytochalasin B, cholera toxin, 12-tetradecanoyl phorbol-13-acetate (TPA), indomethacin, nordihydroguaiaretic acid, antimycin, deoxyglucose, and vanadate were obtained from Sigma Chemical Co. (St. Louis, MO). EGF and nerve growth factor were purchased from Collaborative Research (Lexington, MA); PDGF and acidic FGF were gifts from C.-H. Heldin (Uppsala, Sweden) and M. Jaye (Rorer biotechnology, King of Prussia, US), respectively. Pertussis toxin was from List Laboratories (Campbell, CA); Indo-1 acetoxymethylester and NBD-phallacidin were from Molecular Probes, Inc. (Eugene, OR), 8-Br-cGMP, 8-Br-cAMP, staurosporine, and leupeptin from Boehringer Mannheim Biochemicals (Indianapolis, IN), genistein from Aldrich (Brussels, Belgium), ionomycin and A23187 from Calbiochem-Behring Corp. (La Jolla, CA). Pervanadate was prepared freshly by adding 1 mM H_2O_2 to a 5 mM vanadate solution and incubating the mixture for 15 min at room temperature (Fantus et al., 1989). Excess H_2O_2 was removed by incubation with 200 μg/ml catalase.

Cell Culture

NlE-115 and NG108-15 cells were routinely grown in DME supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), and 10% FCS. For cell rounding assays, cells were seeded at a density of 1-2 × 10^4/cm^2 in plastic 35-mm dishes.

Cell Rounding Assay

Before experiments, low-density cell cultures in 35-mm dishes were shifted to serum-free DMEM for 20–30 h. For cell rounding experiments, dishes were placed on the stage of an inverted microscope (Nikon Diaphot) equipped with phase-contrast optics and temperature control. Cells were maintained in Hepes-buffered DME at 37°C. Agonists and inhibitors were added to the medium from concentrated stocks while the cells were observed continuously. Experiments were documented using a Sony videosystem (type D5CE) connected to a Sony videoprinter (type UP-850) and a Panasonic videorecorder (type AG-6024).

To establish dose-response relationships, cell morphology was determined semi-quantitatively at 3 min after agonist addition. "Percent shape change" was defined as follows: 0%, no detectable change in morphology in the cell population under study (20–40 individual cells); 100%, all previously flattened cells in the population are fully rounded up; and 50%, all cells have changed morphology but are only partially ("half") rounded up. Other values were determined by interpolation. Data are presented as mean ± SEM of experiments performed at least in triplicate.

The kinetics of the cell rounding process was measured by a newly developed optical method, which is based on the property that rounded cells appear more bright than flattened cells when viewed with phase-contrast equipment. Phase brightness was measured using a light-dependent resistor fitted to an eyepiece of the microscope (×20 objective); the light-dependent voltage was low-pass filtered at 1 Hz and recorded on a chart recorder. No attempt was made to calibrate light signals in terms of cell rounding, since there is no simple relationship between phase brightness and cell thickness.

Determination of $[Ca^{2+}]_i$

NlE-115 cells, attached to glass cover slips, were loaded with Indo-1 by exposing them to 5 μM indo-1 acetoxymethylester for 30 min at 37°C. Fluorescence monitoring, calibration procedures, and other experimental details were similar to those described elsewhere (Jalink et al., 1990).

Microinjection of Orthovanadate

NlE-115 cells were microinjected using glass capillary needles (tip ∼0.5 μM) and an automatic pressure-driven microinjection device (Eppendorf/Zeiss, Germany). The microinjected volume was estimated to be <5% of the cell volume. Sodium orthovanadate was dissolved in a microinjection buffer consisting of 100 mM K^+-glutamate and 35 mM K^+-citrate (pH 7.3).

Fluorescence Microscopy

For actin staining, cells grown on circular coverglasses were fixed for 5 min with 3% paraformaldehyde in PBS followed by permeabilization using PBS containing 0.1% Triton X-100 for 3 min. After rinsing in PBS, NBD-phallacidin (2 ng/ml) was incubated with the permeabilized cells for 20 min at room temperature.

Results

Thrombin-induced Neural Shape Changes: a Receptor-mediated Process

In low-density NlE-115 and NG108-15 cultures that have been growth-arrested for 20–24 h, >80% of the cells are flattened often with short (<30 μm) filopodia-like extensions, while 5–10% of the cells have a more fully differentiated morphology, showing elongated neurites (>100 μm). 3–4 d after serum removal all cells assume the fully differentiated morphology.

Addition of thrombin to serum-deprived NlE-115 or NG108-15 cells causes dramatic changes in cellular morphology (Fig. 1). Virtually every flattened cell starts to round up within 10–15 s of thrombin addition, a process that is completed in less than a minute. Concomitantly, developing neurites begin to retract and growth cones collapse, followed by visible degeneration of shrunken neurites (Fig. 1). Thrombin-induced shape changes are dose-dependent with an EC_{50} of ∼0.1 U/ml (Fig. 2); they are completely blocked by hirudin (1 U/ml), a natural and highly specific thrombin inhibitor (Fenton, 1989) as well as by the protease inhibitor leupeptin (10 μg/ml), indicating that thrombin action requires its proteolytic activity.

Strikingly, a novel peptide agonist of the thrombin receptor (SFLLRRNPNDKYEPF), representing the NH_2-terminus of the cleaved receptor (Vu et al., 1991), fully mimics thrombin in inducing cell rounding and neurite retraction with an EC_{50} of 10 μM (Fig. 2). The kinetics and maximum degree of peptide-induced cell rounding, as determined by an optical method, are indistinguishable from those induced by thrombin (Fig. 3). Unlike thrombin, however, the peptide maintains its cell-rounding and neurite-retracting activity in the presence of leupeptin (10 μg/ml; not shown). It thus appears that active thrombin alters neural architecture by directly activating its own G protein–coupled receptor rather than, as previously believed (Monard, 1988), by proteolytically degrading components of the ECM.

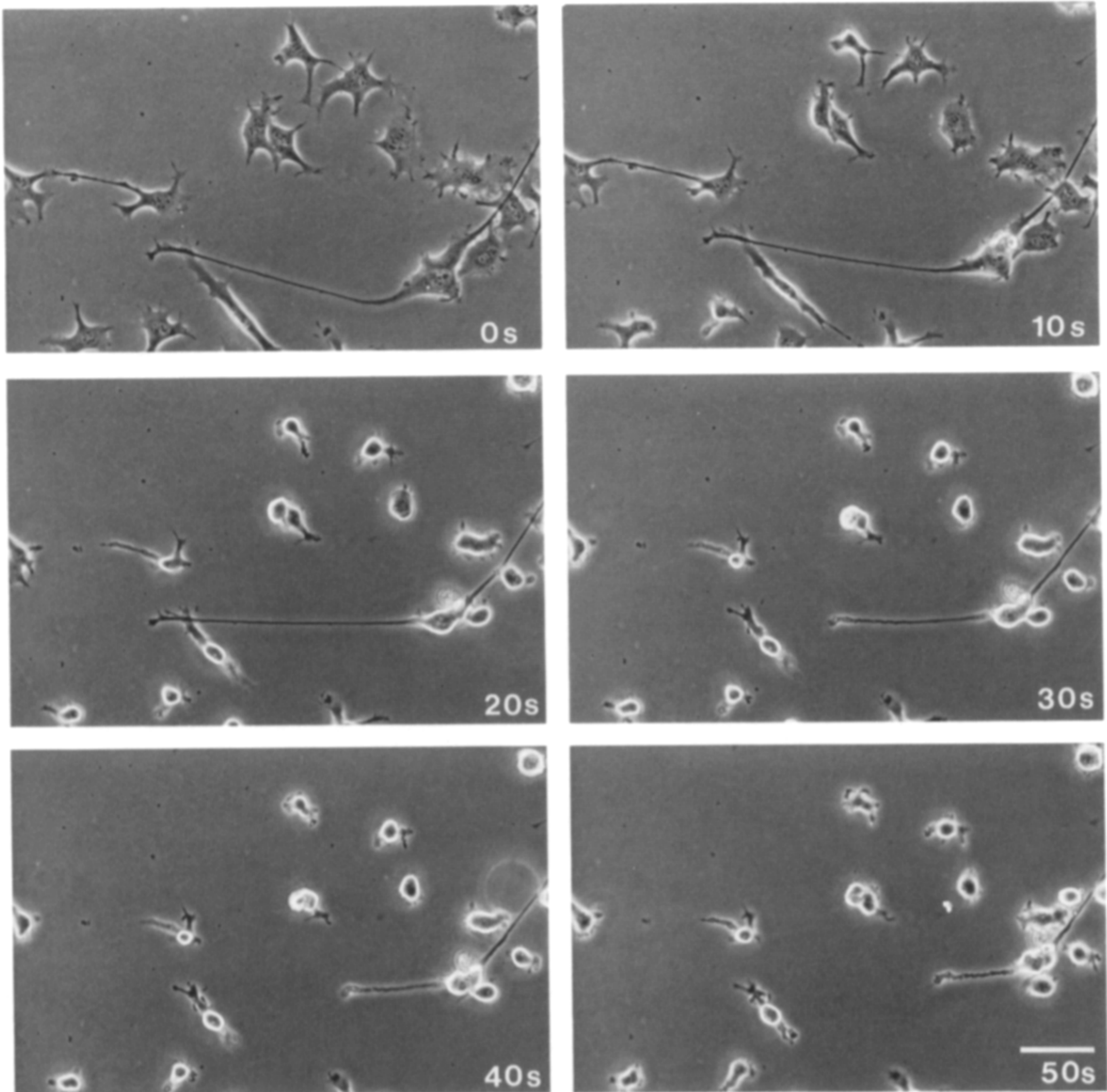

Figure 1. Rapid effects of thrombin (1 U/ml, ~1 nM) on the morphology of serum-deprived N1E-115 cells. Thrombin was added to cells that were deprived of serum for 24 h and cells were photographed at 10-s intervals as indicated. Note the apparent degeneration of shrunken neurites and rapid rounding up of cell bodies. Similar results were obtained with NG108-15 cells. Bar, 50 μm.

Serum Mimics Thrombin

In addition to thrombin receptor agonists, we tested a variety of other ligands and agents for their ability to mimic thrombin in causing neural cell rounding. Serum was found to evoke shape changes very similar to those induced by thrombin or peptide agonist, with half-maximal effects being observed at concentrations of 1–2% vol/vol (Fig. 4). In contrast, a variety of neuropeptides, neurotransmitters and peptide growth factors, Ca^{2+}-ionophores, and protein kinase C–activating phorbol esters all failed to affect neural cell shape within 20 min of their addition (Table I).

What serum factor is responsible for neural cell rounding and neurite retraction? Although serum has been claimed to contain traces of active thrombin (Gurwitz and Cunningham, 1988), the following results strongly argue against a role for thrombin in the morphological response to serum: (*a*) pretreating serum with leupeptin (20 μg/ml) or heating it to 100°C (which destroys thrombin's proteolytic activity) does not affect serum-induced shape changes; (*b*) the response to serum is not attenuated by hirudin (2 U/ml; results not shown); and (*c*) serum does not appear to act through the thrombin receptor as inferred from desensitization experiments described below. Our recent findings suggest that the

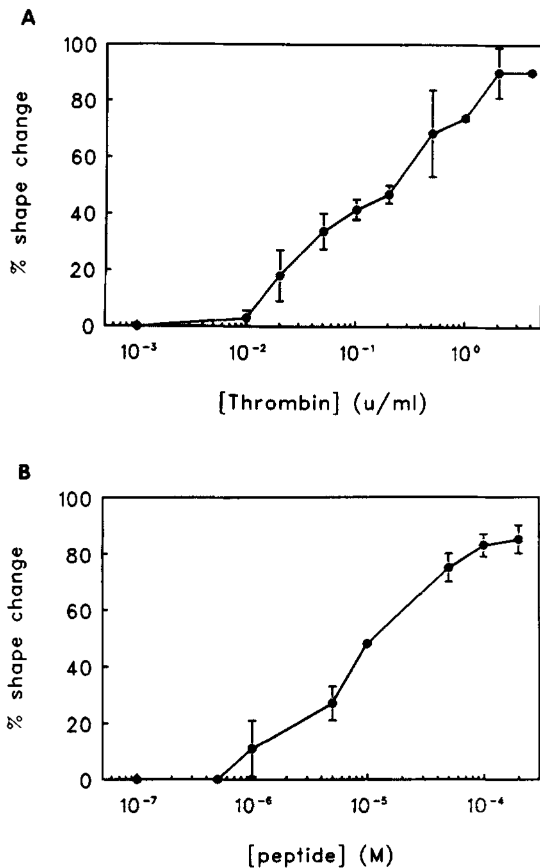

Figure 2. Dose-response relationships of neural shape changes induced by thrombin and a synthetic peptide, SFLLRNPNDKYEPF, mimicking the new amino terminus of the cleaved human receptor. Percent shape changes is defined as described in Materials and Methods. Peptide purity was >95% as revealed by HPLC analysis. Data are presented as mean ± SEM.

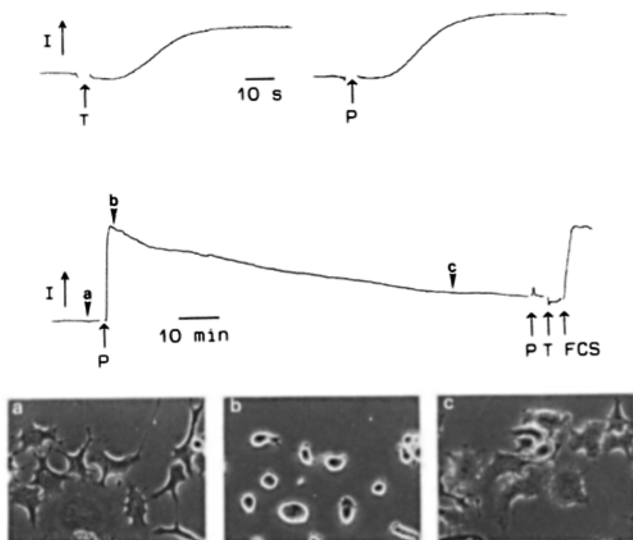

Figure 3. Kinetics of thrombin and peptide agonist-induced shape changes in N1E-115 cells. Cell rounding was monitored by alterations in emitted light intensity (*I*) from a small population of N1E-115 cells (~20 cells) as described in Materials and Methods. T, thrombin (1 U/ml); P, peptide (100 μM); FCS (5% vol/vol). Control peptide, LLRNPNDKYEPF (500 μM), had no effect. Traces show transition from a flattened state (i.e., phase-dark cells) to a fully rounded state (phase-bright cells). *a–c* show cellular morphology corresponding to the indicated intensity of emitted light (*I*). It should be noted that there is no simple relationship between phase-brightness and cell thickness.

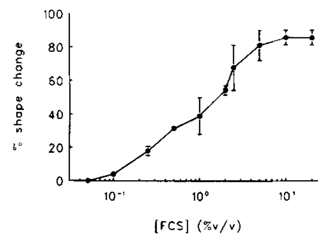

Figure 4. Dose-response relationship for FCS-induced N1E-115 shape changes. Experimental details as described in the legend to Fig. 2 and in Materials and Methods.

shape-changing activity of serum is due, at least in part, to the mitogenic phospholipid lysophosphatidic acid, which seemingly acts via an as yet unidentified receptor (van Corven et al., 1989; Jalink, K., T. Eichholtz, E. J. van Corven, and W. H. Moolenaar, manuscript in preparation).

Reflattening and Homologous Desensitization

In the continuous presence of thrombin or peptide agonist, cells maintain their rounded shape for 10–15 min and then gradually become flattened again (Fig. 3), but neurite outgrowth remains suppressed for prolonged periods of time (>8 h; not shown). After agonist removal, however, neurites reappear after a lag period of several hours (not illustrated). Note that reflattened cells have a completely different morphology when compared to their prestimulation shape. The time-dependent reflattening of thrombin/peptide-treated cells suggests that the response desensitizes. Indeed, readdition of peptide or thrombin to reflattened cells leaves neural cell morphology unaltered, whereas cells do round up again in response to 5% (vol/vol) FCS (Fig. 3). These results indicate that thrombin receptor-mediated cell rounding is subject to homologous desensitization; furthermore, they strengthen the notion that thrombin-induced cleavage of its receptor is sufficient for a full morphological response.

Involvement of the Actin Cytoskeleton

Microtubules and actin microfilaments are the major cytoskeletal elements regulating cell shape. While disruption of these structures by colchicine (10 μg/ml) and cytochalasin B (10 μg/ml), respectively, did not induce rapid N1E-115 cell rounding, cytochalasin B completely blocked thrombin- and peptide-induced cell rounding at a concentration of 0.5 μg/ml. In contrast, colchicine (10 μg/ml) failed to affect cell rounding and neurite retraction (Table I). Fluorescence studies using NBD-labeled phallacidin to stain actin filaments revealed that N1E-115 and NG108-15 cells do not possess well-defined "stress fibers" spanning the cytoplasm, but instead display bright actin staining in the cell periphery only (Fig. 5). In both flattened and rounded cells, actin staining is most prominent in the cell cortex just underneath the plasma membrane (Fig. 5). From these results we

Table I. Agents Tested for Inducing Neural Shape Changes

Agent	Concentration	Cell rounding and neurite retraction
Thrombin	1 U/ml	++
Peptide agonist (SFLLRNPNDKYEPF)	50 μM	++
Serum (fetal or newborn bovine)	5% vol/vol	++
Cytochalasin B + thrombin	0.5 μg/ml + 1 U/ml	−
Colchicine + thrombin	10 μg/ml + 1 U/ml	++
Neurotransmitters (bradykinin, carbachol, endothelin, histamine, serotonin, neurotensin)*	1–100 μM	−
Growth factors (EGF, PDGF, aFGF, NGF)	200 nM	−
Phorbol 12-myristate 13-acetate	100 ng/ml	−
Dioctanoyl glycerol	100 μg/ml	−
Ca^{2+} ionophores (ionomycin; A23187)	1–10 μM	−
Arachidonic acid, oleic acid	10 μM	−
Prostaglandins (PGE_1, PGE_2)	2 μM	−
8Br-cAMP; 8Br-cGMP‡	1 mM	−
Forskolin	10 μM	−
Pertussis toxin; cholera toxin§	200 ng/ml; 10 μg/ml	−
Pertussis toxin + thrombin	200 ng/ml + 1 U/ml	++
Cholera toxin + thrombin	10 μg/ml + 1 U/ml	++

Rounding response of serum-deprived N1E-115 cells was scored semiquantitatively: ++, complete rounding of >80% of the flattened cells within 3 min; −, no detectable cell rounding during a 10-min period at 37°C. Cell culture as described in Materials and Methods. aFGF, acidic FGF; NGF, nerve growth factor.
* Neurotransmitters were selected on basis of their ability to mimic thrombin in mobilizing Ca^{2+} from internal stores in N1E-115 cells.
‡ cAMP-raising agents (8-Br-cAMP, forskolin, cholera toxin) have no early thrombin-like effects on cell shape but tend to stimulate morphological differentiation upon prolonged incubation (>30 min).
§ Toxins were added at least 2 h before testing cellular responsiveness. It was confirmed that both toxins were active by conventional ADP-ribosylation assays (van Corven et al., 1989 and our unpublished data).

conclude that thrombin-induced neural shape changes are mediated by the cortical actin cytoskeleton, with no direct involvement of microtubules.

Signal Transduction Pathways

Classic G protein–dependent signaling events in thrombin action include stimulation of phosphoinositide hydrolysis, arachidonate release and pertussis toxin–sensitive inhibition of adenylate cyclase (e.g., Houslay et al., 1986; Rebecchi and Rosen, 1987; Magnaldo et al., 1988; Siess, 1989). Fig. 6 shows that thrombin, like the peptide agonist, evokes a rapid Ca^{2+} transient that precedes cell rounding; these Ca^{2+} signals persist in the presence of EGTA (3 mM), indicating the release from intracellular stores (not shown). In contrast, Ca^{2+}-mobilizing neuropeptides and neurotransmitters (acting via their cognate G protein-coupled receptors) such as bradykinin, carbachol, endothelin, histamine, serotonin, neurotensin, as well as Ca^{2+} ionophores all fail to affect neural cell shape (Fig. 6 and Table I). What is more, cells depleted of intracellular Ca^{2+} (by addition of ionomycin in Ca^{2+}-free medium) show a normal rounding response to thrombin, despite the absence of a Ca^{2+} signal (Fig. 6). Also, protein kinase C–activating diacylglycerol and phorbol ester leave neural cell shape unaltered (Table I), while staurosporine, at concentrations that preferentially inhibit protein kinase C (1–10 nM), did not affect the rapid morphological effects of thrombin and peptide agonist (not shown; at micromolar concentrations, however, staurosporine is fully inhibitory as shown below).

Together, these results indicate that thrombin-induced phospholipid hydrolysis with consequent Ca^{2+} mobilization and protein kinase C activation is neither necessary nor sufficient for a morphological response. It also can be excluded that thrombin-induced shape changes depend on effectors that couple to pertussis toxin–sensitive G protein(s): under conditions where pertussis toxin stimulates ADP ribosylation of G proteins in N1E-115 cells and prevents receptor-mediated inhibition of adenylate cyclase (van Corven, E. J., unpublished results), we found no effect of the toxin on thrombin action (Table I). Furthermore, preincubation of the cells with cyclic nucleotide-raising agents such as cholera toxin (10 μg/ml), 8-Br-cAMP (1 mM), 8-Br-cGMP (1 mM), or forskolin (10 μM) neither mimicked nor inhibited the morphological response to thrombin. Also, a critical role for phospholipase A_2 seems highly unlikely since free arachidonic acid, prostaglandins (E1 and E2) and potent phospholipase A_2–activating agonists such as bradykinin and ionomycin have no effect on cell shape, while inhibitors of arachidonate metabolism (indomethacin, 5 μM; nordihydroguaiaretic acid, 50 μM) do not block thrombin action (Table I and results not shown).

Finally, there is no apparent role for ionic transmembrane movements in the action of thrombin, since it was observed that thrombin's morphological effects were not affected by eliminating ionic electrochemical gradients using a 130 mM K-glutamate, 10 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 mM Hepes (pH 7.2) incubation buffer (not shown). Therefore, one is left with the conclusion that thrombin receptors bypass conventional second messenger pathways to alter neural architecture.

Inhibition of Thrombin-induced Shape Changes by Kinase Inhibitors, Vanadate, and ATP Depletion

What effector of the thrombin receptor mediates the rapid phenotypic changes? By analogy with the cell rounding induced by active protein tyrosine kinases (Chinkers et al.,

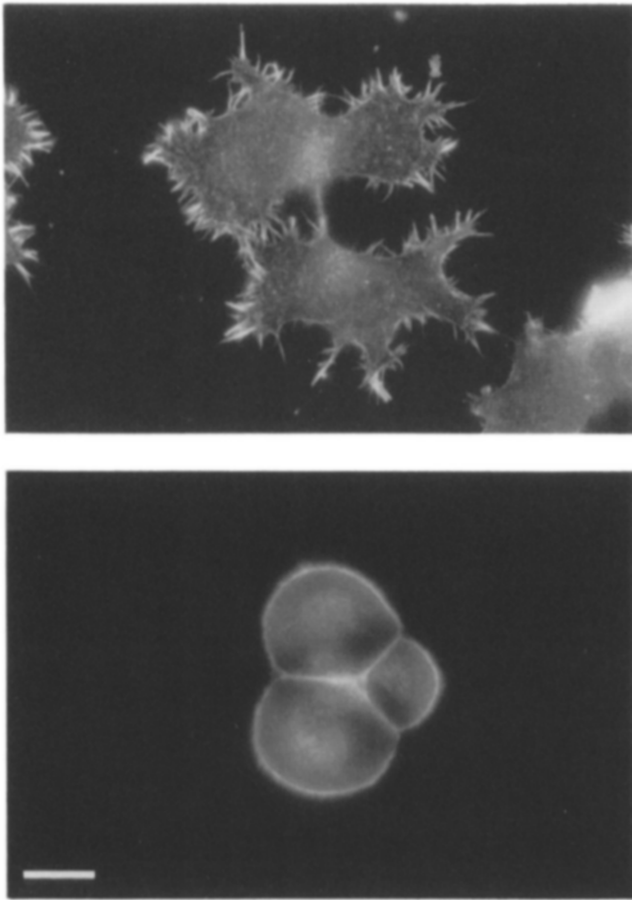

Figure 5. Actin fluorescence in flattened (*upper photomicrograph*) and rounded (thrombin, 1 U/ml, *lower photomicrograph*) N1E-115 cells. Cells were fixed and stained using NBD-phalloidin as described in Materials and Methods. Bar, 30 μm.

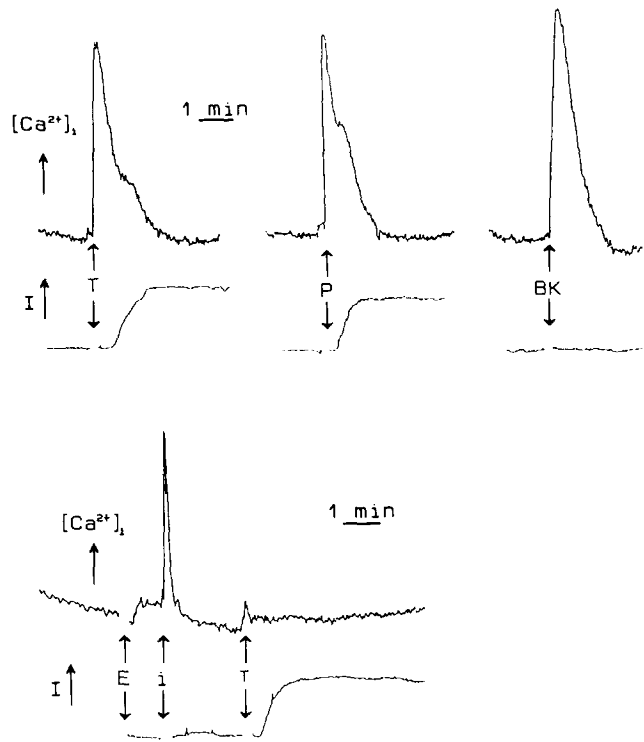

Figure 6. Ca^{2+} signaling and shape changes in N1E-115 cells. Top traces represent typical Ca^{2+} recordings from indo-1-loaded cells; bottom traces are from parallel cell rounding experiments. Arrows indicate time of addition of stimulus. T, thrombin (1 U/ml); P, peptide (100 μM); BK, bradykinin (1 μM); E, EGTA (3 mM); i, ionomycin (5 μM). Intracellular Ca^{2+} concentration and cell rounding (*I*) were measured as described in Materials and Methods. Resting and peak Ca^{2+} levels are close to 150 and 500 nM, respectively.

1981; Jove and Hanafusa, 1987) and microinjected cdc2 kinase (Lamb et al., 1990), it is plausible to assume that specific phosphorylations/dephosphorylations of certain actin-binding proteins (Pollard and Cooper, 1986; Stossel, 1989) may be responsible for the morphological effects of thrombin. In support of this, we observed that thrombin- and peptide-induced shape changes are blocked by brief preincubation of the cells with the broad-specificity protein kinase inhibitors genistein (Akiyama et al., 1987) and staurosporine (Hidaka, 1984; Rüegg and Burgess, 1989) in a dose-dependent manner (Fig. 7 *A*); half-maximal inhibitory concentrations were ~20 μM for genistein and 250 nM for staurosporine. Furthermore, both microinjected orthovanadate, a widely used inhibitor of tyrosine phosphatases and exogenously added pervanadate, which is the membrane-permeable form of vanadate (Fantus et al., 1989), similarly inhibit neural shape changes (Fig. 7 *B*). Finally, no effects of thrombin were observed when cells were depleted of ATP by preincubation (10–15 min) with 0.1 μM antimycin and 10 mM deoxyglucose (not shown). Direct biochemical demonstration of the presumed kinase/phosphatase reactions by which the thrombin receptor communicates to the cytoskeleton awaits further studies.

Discussion

Numerous investigations indicate that neuronal differentiation is governed by a delicate balance between positive and negative regulatory signals. Such diverse extracellular cues as ECM components, integrins, neurotransmitters and secreted proteases and their inhibitors can exert various effects on neuronal architecture, ranging from neurite extension to growth cone collapse and even cell death (for review see Dodd and Jessell, 1988; Monard, 1988; Lipton and Kater, 1989; Doherty and Walsh, 1989; Walter et al., 1990). It is well documented that proteolytically active thrombin can inhibit long-term morphological differentiation in serum-free cultures of spinal cord and brain cells, neuroblastoma cells, astroglia and neuroepithelial cells (Hawkins and Seeds, 1986; Gurwitz and Cunningham, 1988; Monard, 1988; Grand et al., 1989; Nelson and Siman, 1990). Those studies, together with recent reports that prothrombin is synthesized in the brain throughout development (Dihanich et al., 1991), suggest that thrombin may have an important role in neuronal morphogenesis, development, and plasticity. The mechanism underlying thrombin's inhibitory effect on neuronal differentiation is not understood, although most investigators seem to favor the hypothesis that thrombin acts by cleaving proteins involved in cell-substratum attachment, thereby preventing or reversing neurite outgrowth (reviewed by Monard, 1988).

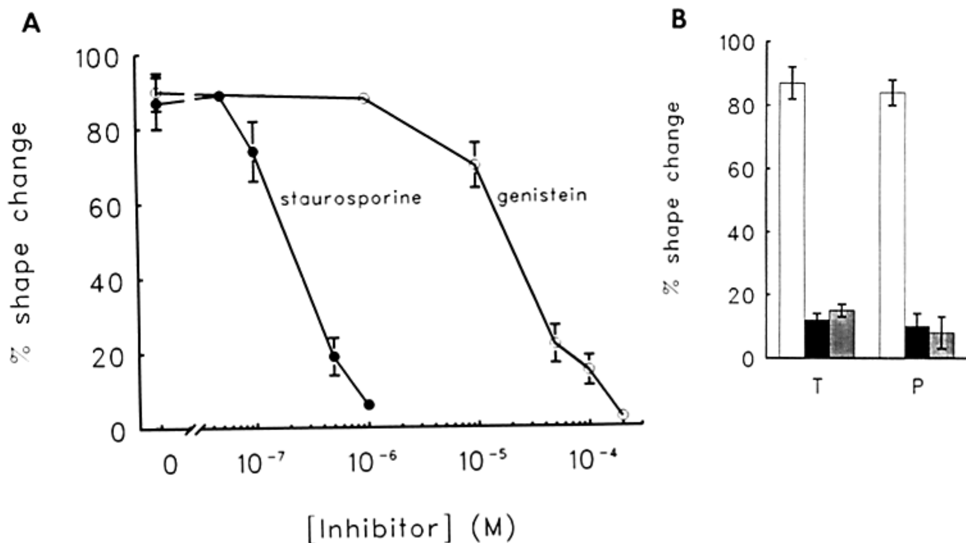

Figure 7. Inhibition of thrombin receptor-mediated shape changes in N1E-115 cells by protein kinase and tyrosine phosphatase inhibitors. (*A*) Cells were preincubated with the indicated concentrations of genistein and staurosporine for 5–10 min and then stimulated with thrombin (1 U/ml) for another 3 min followed by determination of the degree of cell rounding. The inhibitors did not affect cell viability. (*B*) Cells were preincubated with pervanadate (*black bars*) or microinjected with a solution of 5 mM orthovanadate in injection buffer (*hatched bars*) as described in Materials and Methods and assayed for thrombin-induced cell rounding as in *A*. It was confirmed that pervanadate was active as a tyrosine phosphatase inhibitor as follows: N1E-115 cells treated with 100 μM pervanadate for 1–2 min showed a massive increase in tyrosine-specific phosphorylation of cellular proteins as measured in Western blotting experiments using an anti-phosphotyrosine antibody (Verheijden et al., 1990 and our unpublished results). Exogenously added orthovanadate (0.5 mM) had no inhibitory effect (15 min preincubation). Data are presented as mean ± SEM.

In the present study we have observed unprecedented effects of active thrombin on the shape of serum-deprived N1E-115 and NG108-15 neural cells that are in their early stages of morphological differentiation. Thrombin not only triggers neurite retraction followed by disassembly within a few minutes, but also causes the cell body to rapidly round up, as if the cells prepare for mitosis. Rounding is transient, however, and subject to homologous desensitization, whereas neurite outgrowth remains suppressed as long as thrombin is present. Although growth cone collapse and neurite retraction phenomena induced by external stimuli have been reported previously (Lipton and Kater, 1989; Keynes and Cook, 1991), we are not aware of reports of similar rapid and dramatic changes in neural architecture as described here for thrombin-treated N1E-115 and NG108-15 cells under serum-free conditions.

A major result of the present study is that thrombin's morphoregulatory effect is fully mimicked by a novel peptide agonist of the recently cloned thrombin receptor; this peptide, which represents the new amino terminus of the cleaved receptor and obviously lacks proteolytic activity, serves as a tethered ligand for the thrombin receptor and is a full agonist for platelet activation (Vu et al., 1991). Together with the results of desensitization experiments (Fig. 3), it is clear that, at least in N1E-115 and NG108-15 cells, thrombin exerts its morphological effects by activating its own seven-transmembrane-domain receptor, which couples to G proteins, and not through proteolytic attack on ECM components. This does not, of course, rule out the possibility that, in other cell systems, thrombin and thrombin-like proteases can modulate cell shape via a direct action on the ECM rather than via receptor activation. The response to thrombin and peptide agonist is remarkably specific, in that ligands for other G protein–coupled receptors and peptide growth factors all fail to affect neural cell shape. Only serum (5–10% vol/vol) evokes a morphological response that is indistinguishable from that elicited by thrombin. Yet, the effect of serum is not attributable to serum-derived thrombin, since hirudin, leupeptin, and heat inactivation have no detectable effect on the cell-rounding activity of serum. Identification of the serum factor(s) responsible for differentiation reversal remains a focus for further studies; as mentioned in the Results, we have preliminary evidence that the morphological response to serum is attributable, at least in part, to the bioactive phospholipid lysophosphatidate (Jalink, K., T. Eichholtz, E. J. van Corven, and W. H. Moolenaar, manuscript in preparation).

An important question concerns the receptor-mediated biochemical events underlying the morphological response to thrombin. Thrombin receptor–mediated shape changes appear to be due to "contraction" of the actin-based cytoskeleton, with no direct role for microtubules. Indeed, membrane-associated actin filaments, in conjunction with actin-binding proteins, are known not only to serve force-generating and structural roles in nonmuscle cells (Pollard and Cooper, 1986; Bray et al., 1986; Stossel, 1989), but also to participate in the regulation of neurite outgrowth (Smith, 1988). Although intracellular Ca^{2+} has often been regarded as a key regulator of cytoskeletal reorganization in general and of growth cone and neurite behaviour in particular (Kater et al., 1988; Lankford and Letourneau, 1989; Doherty et al., 1991), thrombin action appears to depend neither on Ca^{2+} nor on other classic second messengers such as diacylglycerol and cyclic nucleotides, as outlined under Results and summarized in Table I and Fig. 6. We therefore have to postulate that the thrombin receptor activates an as-yet-uncharacterized effector to reorganize the actin cytoskeleton and to reverse neurite outgrowth. It is currently unknown whether multiple isoforms of the cloned thrombin receptor exist that may activate distinct signaling cascades. It is interesting that another ligand-binding member of the seven transmembrane domain receptor family, the yeast α pheromone receptor, also evokes a "cytoskeletal response" (mating partner discrimination) that is independent of the known G protein–

mediated signal transduction pathways (Jackson et al., 1991). Pharmacologic characterization of the morphological changes suggests that the activated thrombin receptor utilizes vanadate-sensitive phosphatase/kinase cascades to exert its effects. It will be of interest to monitor the activity of *src*-like protein tyrosine kinases in thrombin-treated N1E-115 cells. The *src* kinase, which is highly expressed in neuronal cells, is known to be activated by vanadate-sensitive tyrosine dephosphorylation (Courtneidge, 1985; Bagrodia et al., 1991) and is thought to act on cytoskeletal targets (Jove and Hanavusa, 1987). Moreover, an important role for *src*-like protein tyrosine kinases has been implicated in the action of thrombin on platelets (Huang et al., 1991).

At present, the precise in vivo functions of thrombin and its receptor(s) in the nervous system remain largely unknown. Apart from a normal role in neuronal morphogenesis and synaptic remodeling, thrombin may have a pathophysiological function during traumatic injury of the nervous system when neurons are acutely exposed to blood-borne factors such as thrombin and platelet-derived local mediators. One possibility is that, by rapidly reorganizing their cortical cytoskeleton, blood-exposed neurons may protect themselves from irreversible damage and resume their normal function as soon as thrombolytic activity is antagonized or removed. Regardless of the physiological and pathophysiological functions of thrombin in the nervous system, a challenge for future biochemical studies is to define the sequence of events by which the activated thrombin receptor communicates to the actin-based cytoskeleton in N1E-115 and NG108-15 cells.

After submission of this manuscript, Suidan et al. (1992) reported that thrombin causes neurite retraction in NB2a neural cells through activation of cell surface receptors.

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