Requirement for Rho-mediated Myosin Light Chain Phosphorylation in Thrombin-stimulated Cell Rounding and Its Dissociation from Mitogenesis*

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Thrombin treatment causes a dose-dependent rounding of 1321N1 astrocytoma cells. This cytoskeletal response is rapid, peaking 2 h after thrombin stimulation, and reverses by 50% after 24 h. The thrombin receptor peptide SFLLRNP also induces cell rounding, whereas other G protein-linked receptor agonists such as carbachol, lysophosphatidic acid, or bradykinin fail to do so. Results of studies using pharmacological inhibitors do not support a requirement for phosphatidylinositol 3-kinase, mitogen-activated protein kinase, or Ca2+ mobilization in this response. Inhibition of protein kinase C or tyrosine kinase produces minimal blockade. Pertussis toxin treatment is also without effect. However, thrombin-induced rounding is fully blocked by the C3 toxin from Clostridium botulinum, which specifically ADP-ribosylates and inactivates the small G protein Rho. Thrombin also leads to a rapid, 2.4-fold increase in 32P incorporation into myosin light chain while carbachol does not. Myosin phosphorylation, like cell rounding, is inhibited by inactivation of Rho with C3 exoenzyme, suggesting that myosin phosphorylation is necessary for this cytoskeletal response. This is supported by the observation that thrombin-induced rounding is also blocked by the myosin light chain kinase inhibitor KT5926. However, treatment with KT5926 fails to inhibit mitogenesis. Thus, cell rounding is not prerequisite to thrombin-induced DNA synthesis. We conclude that stimulation of the heterotrimeric G protein-coupled thrombin receptor in 1321N1 cells activates Rho-dependent pathways for both DNA synthesis and cell rounding, the cytoskeletal response being mediated in part through increases in myosin phosphorylation.

Thrombin is a protease known to act through a specific seven-transmembrane spanning G protein-coupled receptor. Thrombin activates its receptor through a proteolytic cleavage mechanism which unmask a new amino terminus that functions as a tethered ligand to activate receptor signaling (1–4). A synthetic peptide analogous to the first five or six NH2-terminal amino acids of the receptor elicits responses similar to thrombin in many cell types (4–8). The best characterized physiological responses to thrombin are its effects on platelet function and DNA synthesis (8–12). Thrombin can also elicit cytoskeletal responses, and has been shown to induce cell rounding in neurons and astrocytes (5, 13, 14). Previous work investigating the mechanism by which thrombin changes cell morphology has shown the process to be activated by the thrombin receptor peptide and thus to be mediated through a cell surface thrombin receptor rather than by other non-receptor-mediated proteolytic mechanisms (5, 14, 15).

In 1321N1 astrocytoma cells, thrombin receptor activation stimulates phospholipase C through Gq, increasing intracellular Ca2+, and activating protein kinase C (PKC)1 (10, 16–18). The muscarinic receptor in 1321N1 cells is also coupled to activation of phospholipase C through Gq, and carbachol elicits changes in phospholipid metabolism, Ca2+ and PKC (16–21), similar to those induced by thrombin. However, while thrombin is mitogenic in astrocytes, carbachol is not (10, 18). This finding and similar observations made in fibroblasts (22) suggest that activation of phosphoinositide hydrolysis by Gq is insufficient for mitogenesis. We recently demonstrated that the novel, PTX-insensitive G12 protein is necessary for thrombin-stimulated DNA synthesis in 1321N1 cells and suggested selective coupling of the thrombin receptor to this G protein (18, 23). The effectors of G12 are not known, but there is some evidence that it transduces its effects via low molecular weight G proteins. We and others have shown that Rac and Rho mediate downstream responses to G12, including activation of mitogen-activated protein kinase cascades and stress fiber formation (24–26).

The Rho family of small GTP-binding proteins is known to be involved in morphological responses and actin cytoskeleton regulation, including formation of actin stress fibers and focal adhesions (27, 28). Several G protein-coupled receptor agonists induce morphological responses which appear to be mediated through Rho. For example, in neuronal cell lines and PC12 cells, LPA has been shown to act through Rho to induce neurite retraction and stress fiber formation (13, 29, 30). Furthermore, Rho has been found to be involved in thrombin-stimulated neurite retraction in both N1E-115 and NG108-15 neuronal cells (13).

These findings imply that receptors known to couple to heterotrimeric G proteins can, at least indirectly, recruit small G proteins of the Rho family and thereby effect cytoskeletal changes. These Rho-dependent changes may be mediated through myosin phosphorylation which regulates the actin- 

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myosin II interaction (31–33) and stabilizes myosin filaments (34, 35) in smooth muscle and non-muscle cells. The ability of GTPγS to increase myosin phosphorylation in smooth muscle cells appears to be mediated through Rho (36–38). Kaibuchi and others (39) have shown that activated Rho increases myosin phosphorylation through Rho kinase, which phosphorylates the myosin-binding subunit of myosin phosphatase, inhibiting its activity and increasing levels of phosphorylated myosin. Rho kinase has also been shown to directly phosphorylate myosin light chain at the same site as myosin light chain kinase (40). Other recently described Rho-binding proteins such as protein kinase N and phosphatidylinositol 4,5-kinease (41, 42) could also mediate Rho-dependent effects on cell morphology.

We have utilized the 1321N1 astrocytoma cell line to examine the molecular mechanisms that underlie actin cytoskeletal changes induced by thrombin, specifically the involvement of second messengers, Rho, and myosin phosphorylation in this response; related studies have also determined whether the thrombin-induced morphological change is required for mitogenic responses to this agonist. We demonstrate here that thrombin induces marked changes in cell morphology which, like DNA synthesis, are dependent on Rho function. Cell rounding can be dissociated from DNA synthesis, however, and appears to be mediated via Rho-dependent increases in the phosphorylation state of myosin light chain.

EXPERIMENTAL PROCEDURES

Cell Culture—Human 1321N1 astrocytoma cells were plated onto 100-mm plates at a density of 1.2 × 10^6 cells/ml and grown in Dulbecco’s modified Eagle’s media (DMEM) supplemented with 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) for 4 days. The cells were then trituated and set on 12-mm round glass coverslips at 1.5 × 10^5 cells/lipid and allowed to grow in 5% fetal calf serum + DMEM overnight to assay rounding. DNA synthesis was quantified by performing Ca^2+ imaging with fura-2/AM, as described below.

Rounding (Reversal of Stellation) Assay—Cells on coverslips were transferred to 35-mm plastic dishes containing serum-free DMEM. Cells were then treated with 0.5 unit/ml (12 nM) thrombin (Calbiochem), as the thrombin-peptide, SFFLRNP (100 μM) (synthesized at Immologic, San Diego), and fixed at various times thereafter in 3.7% formaldehyde/phosphate-buffered saline. In studies examining the effects of different inhibitors on astrocyte morphology, cells were preincubated with agents (e.g. forskolin, isobutylmethylxanthine, hermbicyn, KT5926, etc.) for the desired time, after which the cells were stimulated with thrombin for 2 h. Following fixation, the cells were permeabilized with 0.5% Triton X-100/phosphate-buffered saline and stained with rhodamine-conjugated phallolidin (Molecular Probes) to visualize actin filaments. Cells were examined by fluorescence microscopy and at least 300 cells scored for rounding under each condition.

DNA Synthesis Assays—To measure DNA synthesis, cells were set on coverslips as described above and serum-starved for 24 h. The coverslips were then transferred to 35-mm plastic dishes containing serum-free DMEM and stimulated with thrombin for 24 h in the presence of BrdUrd. Cells were then fixed in 95% ethanol and 5% glacial acetic acid, and immunostained for BrdUrd incorporation using a mouse monoclonal anti-BrdUrd antibody (Amersham) and a secondary rhodamine-conjugated goat anti-mouse antibody (Cappel). Cells were visualized using a Zeiss-Axiophot microscope and a 40 × Neofluor objective. DNA synthesis was quantified by scoring the percentage of cells with BrdUrd-positive nuclei in a field of approximately 200 cells.

In some experiments DNA synthesis was examined by measuring [3H]thymidine incorporation. Cells were set on 6-well plates at a density of 2.4 × 10^5 cells/well and grown in DMEM containing 5% fetal calf serum for 24 h. Cells were then shifted to serum-free DMEM plus 0.1% bovine serum albumin (essentially fatty acid free) for 48 h before stimulation with thrombin. DNA synthesis assays with the MLCK inhibitor were performed by precubication with 100 nM KT5926 for 2 h before stimulation with thrombin. Cells were then treated with thrombin for 24 h, and incorporation of [3H]thymidine into DNA was measured with 2 μCi/ml [methyl-3H]thymidine which was included for the last 6 h of the incubation at 37 °C. Cells were then washed twice with cold phosphate-buffered saline and fixed in cold methanol for 10 min. Unincorporated precursor was removed in 2 washes of ice-cold 10% trichloroacetic acid (10 min), and 1 wash of cold 0.5% trichloroacetic acid (5 min). The monolayer was then dissolved in 1 ml of N NaOH for 30 min at room temperature. 1 ml of 1 N HCl and scintillation fluid was added to the monolayer and the mixture was counted on a Beckman LS 5000 CE scintillation counter.

C3 Toxin Fusion Protein and Expression Plasmid—cDNA for the GST-C3 fusion protein (kindly provided by Dr. J. Meinkoth, University of Pennsylvania) was used to transform JM109 Escherichia coli to produce the GST-C3 fusion protein for purification. After transformation, the cells were lysed and clarified extracts incubated with GSH-Sepharose. Following washing, the C3 toxin protein was eluted from the GST by overnight incubation with thrombin. Thrombin was removed by incubation with para-aminobenzamidine-Sepharose, and the supernatant was concentrated in a Centricron-10 to a final concentration of 5 mg/ml protein. The expression plasmid for the C3 transferase (EFC3) was generously provided by Dr. Richard Treisman (ICRF, London). 1321N1 cells transfected with the EFC3 plasmid by coprecipitation of C3 PO4 were identified by luciferase immunostaining and the rounding response was quantified by fluorescence microscopy.

Ca^2+ Measurements in 1321N1 Cells with Fura-2/AM—Intracellular calcium concentrations were measured using the fluorescent calcium indicator fura-2. 1321N1 cells were set on glass coverslips as described previously, and grown overnight in 5% fetal calf serum + DMEM. Cells were then treated with fura-2/AM (2 μM) by incubating in 5 μM fura-2/AM at 37 °C with 3 μm fura-2/AM and with or without BAPTA/AM for 20–30 min. The cells were then washed and changes to fura-2 free DMEM and fluorescence were monitored at 350 and 380 nm.

Myosin Phosphorylation—1321N1 cells were plated in DMEM containing 5% fetal calf serum on 10-cm plates at a density of 3 × 10^5 cells/ml. After 3 days, cells were washed with serum-free and PO4-free DMEM, changed to 5 ml of PO4-free DMEM, and labeled with 50 μCi/ml [3H]thymidine for 3 h. In some experiments cells were pretreated with C3 exoenzyme (40 μg/ml), KT5926 (100 nM), or BAPTA/AM (20 μM) for 10–16 h, 3 h, or 30 min, respectively, prior to agonist addition. Following 5 min of stimulation with 12 nM thrombin (0.5 μM) or 0.5 mM carbachol, the assay was terminated by washing cells with cold phosphate-buffered saline, freezing on dry ice, scraping cells into an extraction buffer (50 mM pyprophosphate, 100 mM sodium fluoride, 300 mM NaCl, 10 mM NaNO3, 10 mM EGTA, 2 mM EDTA, 2.5 mM PO4, 1% Nonidet P-40, pH 7.4), and centrifugation at 20,000 rpm for 20 min. Myosin protein was immunoprecipitated using an antibody to macrophage myosin II heavy chain (43). The immunoprecipitates were resuspended in 0.25% SDS, 5 mM dithiothreitol, 5 mM Tris, pH 6.8, boiled, and run on 12% SDS-PAGE gels to separate myosin light and heavy chains. Myosin light chain phosphorylation was quantitated on an Ambis radioanalytic scanner.

RESULTS

Thrombin Induces Characteristic Cell Rounding and Mitogenesis in Astrocytoma Cells—Addition of thrombin to 1321N1 human astrocytoma cells produced a concentration dependent cell rounding (reversal of stellation) which was maximal (∼90% of cells) at 0.5 unit/ml (12 nM) (Figs. 1A and 2A). The thrombin receptor peptide, SFFLRNP, also induced rounding, although its effect was more variable than that of thrombin (Fig. 2A). In contrast, cells stimulated with carbachol (Figs. 1C and 2A) retained the stellate unrounded morphology characteristic of control cells (Fig. 1A). Two other G protein-coupled receptor agonists, LPA (10 μM) and bradykinin (1.0 μM), were unable to induce 1321N1 cell rounding (not shown).

Thrombin is an efficacious mitogen for 1321N1 cells causing a marked increase in the number of cells synthesizing DNA (10, 18) (Fig. 2B). The thrombin-receptor peptide (SFFLRNP) also stimulated BrdUrd incorporation (Fig. 2B). In contrast, carbachol, LPA, and bradykinin treatment did not induce mitogenesis (Fig. 2B and data not shown), although these agonists, like thrombin, were able to activate phosphorylation of the C3 toxin protein in Ca^2+ mobilization (17, 18, 29, 44, 45). Thus activation of the thrombin receptor by thrombin or the tethered ligand peptide has unique effects on 1321N1 cell morphology and DNA synthesis which are not shared by agonists for other phospholipase C-linked G protein receptors.

Time Course and Dose-response Relationship for Thrombin-
induced Rounding—The time course of thrombin-induced cell rounding is shown in Fig. 3. Rounding is observed within 1 min and virtually all cells are rounded 1–2 h after stimulation. The cell rounding is transient and begins to reverse after 2 h in the continued presence of thrombin. The dose-response curves for thrombin-induced rounding (assessed at 2 h when maximal rounding is seen) and \[^{[3]}H\]thymidine incorporation (assayed at 24 h when DNA synthesis is maximal) are nearly superimposable (Fig. 4). The EC\textsubscript{50} for thrombin-induced rounding was 0.15 nM while that for \[^{[3]}H\]thymidine incorporation was determined to be 0.55 nM.

Effect of Kinase Inhibition on Thrombin-induced Rounding—To examine the mechanism by which thrombin induces shape changes in 1321N1 cells we determined whether this response was sensitive to specific kinase inhibitors (Fig. 5). Treatment with 100 nM wortmannin, a phosphatidylinositol 3-kinase inhibitor, did not block thrombin-induced rounding. This concentration of wortmannin has previously been shown to inhibit insulin stimulation of phosphatidylinositol 3-kinase activation in 1321N1 cells (46). The protein kinase C inhibitor GF109203X, at 1 μM, likewise only partially inhibited cell rounding; higher concentrations (5–10 μM) did not inhibit further. This same concentration of GF109203X was able to inhibit thrombin-induced AP-1 expression and mitogenesis as measured by \[^{[3]}H\]thymidine incorporation in 1321N1 cells (data not shown). Treatment with 1 μM herbimycin A or another tyrosine kinase inhibitor, genistein (data not shown), led to only modest decreases in thrombin-induced rounding. Thrombin activates the Ras and MAP kinase mitogenic cascade in 1321N1 cells (10, 18). However, this pathway is apparently not required for thrombin-induced rounding, since the MAP kinase kinase inhibitor PD098059 (47) at 50 μM had no effect on cell rounding. Herbimycin, genistein, and PD098059, at the concentrations used here inhibited thrombin-stimulated DNA synthesis in 1321N1 cells (data not shown).

Effect of Pertussis Toxin on Morphological Response to Thrombin—The cloned thrombin receptor can interact with multiple heterotrimeric G proteins to transduce intracellular signals. To examine involvement of G/G, proteins in thrombin-induced morphological effects, cells were treated with pertussis toxin (PTX), alone or in combination with thrombin, for 1 h. Cells were then washed and cultured in the presence of PTX and thrombin. As shown in Fig. 6, PTX blocked thrombin-induced rounding, suggesting the involvement of a G protein in this process. The effect of PTX was specific for G proteins, since PTX had no effect on thrombin-induced mitogenesis (data not shown). These results suggest that G proteins, possibly G\textsubscript{i} and G\textsubscript{q}, are involved in the morphological response to thrombin in 1321N1 cells.
Effects of kinase inhibitors on thrombin-induced rounding. 1321N1 astrocytoma cells were preincubated in serum-free media with 100 μM wortmannin (Wort) for 1 h, 1 μM herbinycin A (HerbA) for 15 min, 50 μM PD098059 (PD), or 30 μM GF109203X (GF), for 15 min, and then stimulated for 2 h with vehicle (white bars) or thrombin (black bars). Data are expressed as the mean ± S.E. from two separate experiments done in duplicate in which approximately 200 cells were counted on each coverslip.

Ca²⁺ Requirement for Thrombin-induced Rounding—The thrombin receptor in fibroblasts and 1321N1 cells couples via a Gₛ-dependent pathway to stimulate phosphoinositide hydrolysis and mobilize intracellular Ca²⁺ (16, 18, 49). To test the possible involvement of increased cytosolic Ca²⁺ in thrombin-induced rounding, cells were pretreated with 30 μM BAPTA/AM, which was previously shown to prevent the carbachol-induced rise in intracellular Ca²⁺ in 1321N1 cells (21). BAPTA/AM pretreatment did not decrease thrombin-induced cell rounding (Table I). To confirm that the BAPTA/AM effectively blocked thrombin-induced intracellular Ca²⁺ release, we used Ca²⁺ imaging of 1321N1 cells loaded with fura-2/AM. Cells loaded with fura-2/AM and pretreated with BAPTA/AM showed no Ca²⁺ spike upon stimulation with 12 nM thrombin or 200 μM carbachol (data not shown). Thus, thrombin-mediated retraction of processes can occur in the complete absence of increases in cytosolic free Ca²⁺. In addition, chelation of extracellular Ca²⁺ by addition of 3 mM EGTA to the medium (Table I) or incubation in Ca²⁺-free medium (not shown) failed to inhibit thrombin-induced rounding.

Involvement of Rho in Thrombin-induced Rounding—The involvement of the small G protein Rho in thrombin-induced rounding of 1321N1 cells was examined by treating cells with the C3 exoenzyme from Clostridium botulinum, which specifically and irreversibly ADP-ribosylates Rho (13, 50–52). Treatment of cells with 40 μg/ml C3 toxin alone for 10–16 h had no apparent effect on cell morphology; however, these cells no longer developed a rounded morphology in response to stimulation with thrombin (Fig. 6A). Partial inhibition was seen in cells pretreated with a lower concentration (20 μg/ml) of C3 toxin (data not shown). The observed effect of C3 toxin was confirmed using an expression plasmid for the C3 transferase (EFC3). Cells transfected with the C3 toxin expression plasmid showed a marked decrease in thrombin-stimulated rounding which was dependent on the amount of EFC3 plasmid transfected (data not shown).

To determine whether Rho function was also required for thrombin-induced mitogenesis, we pretreated 1321N1 cells with 40 μg/ml C3 toxin for 10–16 h and quantitated BrdUrd incorporation after thrombin stimulation. C3 toxin treatment markedly inhibited BrdUrd incorporation, thus implicating Rho in thrombin-stimulated DNA synthesis as well as cell rounding (Fig. 6B).

Effect of Myosin Light Chain Phosphorylation on Thrombin-induced Rounding—Since it is known that the enzyme myosin light chain kinase regulates actin-myosin force generation in smooth muscle contraction and other contractile responses, we examined its involvement in thrombin-induced cell rounding. Treatment with the myosin light chain kinase inhibitor, KT5926, caused a dose-dependent inhibition of rounding with complete inhibition at 100 nM (Fig. 7A). Since KT5926 can also inhibit CaM kinase II, we compared the effects of KT5926 to those of a specific CaM kinase inhibitor, KN-93. KN-93 did not significantly decrease thrombin-induced rounding even at concentrations up to 2 μM, five times its Kᵢ for inhibition of CaM kinase II (data not shown). Thus inhibition of CaM kinase does not prevent cell rounding and is not likely to contribute to the inhibitory effect of KT5926.

In contrast to the marked inhibitory effect of KT5926 on cell rounding, KT5926 had no effect on thrombin-stimulated motogenesis as measured by [³H]thymidine incorporation (Fig. 7B) or BrdUrd incorporation (data not shown). To rule out the possibility that the inhibitor was degraded, or lost activity during the 24 h required to assess DNA synthesis, KT5926 was re-added at 10 h after thrombin addition; no decrease in [³H]thymidine incorporation was observed. To further test the stability of KT5926 we pretreated cells with the inhibitor for
10 h prior to stimulation with thrombin. KT5926 pretreatment completely blocked thrombin-induced rounding, indicating that the inhibitor remained active. These data establish that thrombin-induced mitogenesis does not require myosin light chain kinase and, significantly, that the effects of thrombin on cell rounding and DNA synthesis can be dissociated.

Regulation of Myosin Light Chain Phosphorylation—We assessed phosphorylation of myosin in 32P-labeled cells to examine the possible involvement of myosin phosphorylation in thrombin-induced cell rounding. Myosin was immunoprecipitated using an antibody to myosin heavy chain, the immunoprecipitates separated by SDS-PAGE and myosin light chain phosphorylation quantitated by radiometric scanning. Thrombin stimulation increased the amount of 32P incorporated into myosin by 2.4-fold over unstimulated cells (Fig. 8, A and B). Carbachol, in contrast, did not lead to increased myosin light chain phosphorylation. Blockade of Rho function with C3 exoenzyme pretreatment inhibited the thrombin-stimulated increase in myosin light chain phosphorylation. Thus, thrombin treatment was directly shown to increase the phosphorylation state of myosin light chain, and this response shown to depend upon Rho function. The role of MLCK in the phosphorylation of myosin light chain was also tested. KT5926 and BAPTA/AM treatment led to a modest inhibition of myosin light chain phosphorylation (Table II), suggesting that MLCK is not the predominant pathway regulated by thrombin.

Fig. 6. Effect of C3 exoenzyme pretreatment on thrombin-induced rounding and DNA synthesis. A, 1321N1 cells were pretreated with 40 μg/ml C3 exoenzyme (prepared as described under “Experimental Procedures”) for 10–16 h before stimulation with thrombin (0.5 unit/ml) for 2 h. Morphology was determined using rhodamine-phalloidin staining as described. Unstimulated cells which were preincubated with C3 exoenzyme showed normal processes. Data are expressed as mean ± S.E. from three experiments. B, effect of C3 toxin on DNA synthesis was determined by preincubation with 40 μg/ml C3 exoenzyme protein for 10–16 h and percent BrdUrd incorporation was visualized after thrombin stimulation for 24 h as described previously.

Fig. 7. Effect of myosin light chain kinase inhibitor, KT5926, on thrombin-induced rounding and mitogenesis. A, 1321N1 cells were pretreated with varying concentrations of the MLCK inhibitor KT5926 in the absence of serum for 2 h prior to stimulation with thrombin (0.5 unit/ml) for 2 h. Data are means of two experiments or mean ± S.E. from three or more experiments. B, 1321N1 cells were pretreated with 100 nM KT5926 for 2 h before stimulation with thrombin (0.5 unit/ml) overnight and DNA synthesis assessed by [3H]thymidine labeling or BrdUrd incorporation (data not shown). Data from [3H]thymidine counts/min is expressed as fold stimulation over each respective basal and is the mean ± S.E. of six separate experiments.

DISCUSSION

The data presented here demonstrate that thrombin treatment leads to a pronounced cytoskeletal response, characterized as cell rounding or reversal of stellation, in 1321N1 astrocytoma cells. Morphological responses to thrombin were once considered to result from proteolytic effects on extracellular matrix proteins (15, 53), but as we show here, and others have recently demonstrated, the thrombin receptor peptide SFLLRN mimics this effect of thrombin. The receptor peptide lacks proteolytic activity, acting instead like the tethered ligand shown to be formed when the cloned thrombin receptor is activated by thrombin. Thus this cytoskeletal response appears to be regulated through ligand interaction with the cell surface thrombin receptor.

The thrombin receptor is a G protein-coupled receptor which is capable of both inhibiting adenylate cyclase through G(i) and stimulating phospholipase C through Gq or other PTX-insensitive G protein(s) (11). DNA synthesis in fibroblasts is regulated through the same cloned receptor (54). We have shown that this receptor is also expressed in 1321N1 cells,2 where thrombin regulates phospholipase C (16), AP-1 mediated gene expression (55), and DNA synthesis (10) through PTX-insensitive pathways (18). Thrombin-induced cell rounding, like these other responses, is PTX-insensitive, consistent with results obtained from studies examining thrombin- and LPA-induced cell rounding in neuronal and PC-12 cells (14, 56). Thus, neither α nor βγ subunits from the pertussis toxin-sensitive G(i)/G(o) family G proteins appear to mediate this response.

2 D. Goldstein, and J. H. Brown, unpublished results.
were stimulated with thrombin (0.5 unit/ml) or carbachol (0.5 mM) for 5 min. Myosin was immunoprecipitated using an antibody against macrophage myosin II heavy chain and myosin light chain separated by SDS-PAGE. Dried gels were quantitated on an Ambis radiometric scanner. Data in A are from two representative experiments. Data in B are mean ± S.E. of values normalized to the unstimulated control and are pooled from separate experiments (control and thrombin, n = 7; C3 pretreated, n = 4; carbachol, n = 2).

**Table II**

Effects of the MLCK inhibitor KT5926 and the intracellular calcium chelator BAPTA/AM on thrombin-stimulated myosin light chain phosphorylation

| Treatment          | Counts/min | Fold  |
|-------------------|------------|-------|
| Control           | 189.23 ± 17.2 | 1     |
| Thrombin          | 450.14 ± 43.5 | 2.4   |
| Thrombin + BAPTA/AM | 326.15 ± 41.5 | 1.7   |
| Thrombin + KT5926 | 392.5 ± 55.2 | 2.1   |

**Fig. 8.** Effect of thrombin, carbachol, and C3 exoenzyme on myosin light chain phosphorylation. 32P-Labeled 1321N1 cells were stimulated with thrombin (0.5 unit/ml) or carbachol (0.5 mM) for 5 min, or pretreated with C3 exoenzyme (40 μg/ml) for 10–16 h before thrombin stimulation for 5 min. Myosin was immunoprecipitated using an antibody against macrophage myosin II heavy chain and myosin light chain separated by SDS-PAGE. Dried gels were quantitated on an Ambis radiometric scanner. Data in A are from two representative experiments. Data in B are mean ± S.E. of values normalized to the unstimulated control and are pooled from separate experiments (control and thrombin, n = 7; C3 pretreated, n = 4; carbachol, n = 2).

**Fig. 9.** Proposed pathways leading to thrombin-induced myosin phosphorylation and cell rounding. Bold arrows indicate the pathway postulated to predominate in mediating thrombin-induced cell rounding.

Several lines of evidence also indicate that activation of phospholipase C and generation of its second messengers does not account for the PTX-insensitive cytoskeletal effects mediated by thrombin receptor activation. First, we show here that pretreatment with BAPTA-AM, which blocks the agonist and pharmacological inhibition of PKC causes only a partial decrease in thrombin-stimulated myosin phosphorylation. Blocking the thrombin-stimulated rise in intracellular Ca2+ with BAPTA/AM also leads to only a modest decrease in myosin phosphorylation and cell rounding.

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Rho-mediated Myosin Phosphorylation in Cell Rounding

There are several downstream effectors of Rho which could mediate cell rounding or stress fiber formation. One candidate is the phosphatidylinositol 4,5-kinase which was reported to be regulated by Rho in fibroblasts (42). This enzyme regulates the levels of polyphosphoinositides, which can bind to and control myofilament-protein interactions (59, 60). Additionally, Rho has been shown to interact directly with and activate serine/threonine kinases among which is protein kinase N, a PKC-related kinase (41). The most likely candidate for the effector responsible for Rho-mediated cell rounding, however, is the Rho-dependent kinase. This enzyme has been shown to mediate stress fiber and focal adhesion formation in fibroblasts (61) and to regulate myosin light chain phosphorylation (40, 62).

A simplified schematic of pathways which can regulate myosin light chain phosphorylation is presented in Fig. 9. The best characterized enzyme involved in myosin phosphorylation is the Ca2+-calmodulin-dependent enzyme MLCK. Phosphorylation of myosin light chains by MLCK stimulates the actin-activated ATPase in smooth muscle and non-muscle myosin (31–33). Myosin phosphorylation has been shown to regulate smooth muscle contractility (31–33) and non-muscle cell responses such as cell motility (63, 64), epithelial barrier function (65, 66), and endothelial contractility (67). In addition to MLCK, myosin light chain can be phosphorylated by PKC, PKA, and PAK (p21-activated kinase 1) (68–70), although the physiological significance of these phosphorylation reactions is unclear. Most recently, myosin phosphorylation has been shown to be increased by Rho-dependent pathways involving phosphorylation of the myosin-binding subunit of myosin phosphatase (39) and direct phosphorylation of myosin light chain (40, 62).

Myosin phosphorylation does not appear to be mediated solely through thrombin-induced activation of MLCK. As we demonstrate here, inhibition of MLCK with KT5926 produces only a partial decrease in thrombin-stimulated myosin phosphorylation. Blocking the thrombin-stimulated rise in intracellular Ca2+ with BAPTA/AM also leads to only a modest de-
crease in myosin light chain phosphorylation. Furthermore, carbachol, which mobilizes intracellular Ca\(^{2+}\) and should activate MLCK to the same extent as thrombin, does not increase myosin phosphorylation at the time point examined in our experiments. These findings indicate that additional pathways are involved in regulating the state of myosin phosphorylation in thrombin-stimulated 1321N1 cells. The observation that C3 exoenzyme fully inhibits thrombin stimulation of myosin light chain phosphorylation suggests that a Rho-dependent pathway predominates in regulating myosin phosphorylation in this system.

Thrombin-induced cell rounding is a Rho-dependent phenomenon which we suggest is mediated via myosin phosphorylation. The Ca\(^{2+}\)-dependent pathways that can presumably lead to myosin phosphorylation do not appear necessary to induce this cytoskeletal response, since BAPTA/AM does not prevent cell rounding. On the other hand, inhibition of Rho with C3 exoenzyme fully blocks cell rounding, implicating Rho-dependent myosin phosphorylation. Evidence from other systems suggests that Ca\(^{2+}\)-dependent activation of MLCK is important for rapid increases in myosin light chain phosphorylation such as those required for smooth muscle contraction. In contrast, Rho-dependent pathways appear to result in sustained increases in myosin phosphorylation which, in turn, have been suggested to change the calcium sensitivity of smooth muscle contraction (37, 38, 71).

We suggest that thrombin-induced rounding likewise involves a more sustained increase in myosin phosphorylation. Cell rounding peaks at 2 h of continuous thrombin stimulation, and removal of thrombin during this 2-h period prevents maximal rounding. A sustained increase in myosin phosphorylation would be best achieved through simultaneous inhibition of myosin phosphatase and activation of myosin kinases. Thrombin may utilize both of these mechanisms by eliciting Rho kinase-dependent activation of myosin phosphorylation and inhibition of myosin phosphatase. The effect of thrombin on the actin cytoskeleton may be dissociated from rapid, transient, Ca\(^{2+}\)-mediated increases in myosin phosphorylation and thus insensitive to BAPTA. However, development of a maximal response to thrombin may be inhibited by KT5926 if the basal rate of myosin phosphorylation is not maintained. Alternative effects of KT5926, involving mechanisms other than MLCK or calmodulin kinase inhibition are also possible.

A relationship between the morphological and mitogenic effects of thrombin is suggested by several observations. One is that carbachol and bradykinin, which do not induce rounding in 1321N1 cells, also fail to stimulate DNA synthesis. In contrast, thrombin and the thrombin receptor peptide induce both responses and the two effects of thrombin occur over a similar range of concentrations. Furthermore, 1321N1 cells stably expressing P2Y1 purinergic receptors (72), which are not present in wild type cells, undergo both rounding and mitogenesis in response to ATP, associating the occurrence of these two responses. Finally, rounding and DNA synthesis were both inhibited by C3 exoenzyme, suggesting that these events could use a common Rho-dependent signaling pathway. Despite the seemingly parallel relationship between rounding and DNA synthesis, however, rounding is not obligatory for DNA synthesis, since KT5926 inhibits thrombin-induced rounding but does not affect mitogenesis. Moreover, these data suggest that thrombin activates multiple Rho-dependent signaling path-

ways, one leading to rounding, another involving downstream effectors of Rho required for DNA synthesis.

The studies presented here demonstrate that activation of the heterotrimeric G protein-coupled thrombin receptor induces a cytoskeletal response characterized by cell rounding. We provide evidence that thrombin stimulates myosin light chain phosphorylation, and that both Rho activation and myosin light chain phosphorylation are required for changes in cell morphology. The physiological significance of the cytoskeletal response to thrombin in astrocytes remains to be determined. These shape changes may be important in normal brain processes (e.g. in cell differentiation or migration during development or neural plasticity), or in response to brain injury (e.g. in wound healing or gliosis) (5, 29). The effects of thrombin on astroglial cell function demonstrate that a subset of G protein-coupled receptors can control Rho-dependent increases in myosin light chain phosphorylation regulating the actin cytoskeleton.

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Rho-mediated Myosin Phosphorylation in Cell Rounding

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