Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways

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Am J Physiol Cell Physiol 283: C1351–C1364, 2002. First published June 20, 2002; 10.1152/ajpcell.00001.2002.—Protease-activated receptors (PARs), newly identified members of G protein-coupled receptors, are widely distributed in the brain. Thrombin evokes multiple cellular responses in a large variety of cells by activating PAR-1, -3, and -4. In cultured rat astrocytes we investigated the signaling pathway of thrombin- and PAR-activating peptide (PAR-AP)-induced cell proliferation. Our results show that PAR activation stimulates proliferation of astrocytes through the ERK pathway. Thrombin stimulates ERK1/2 phosphorylation in a time- and concentration-dependent manner. This effect can be fully mimicked by a specific PAR-1-AP but only to a small degree by PAR-3-AP and PAR-4-AP. PAR-2-AP can induce a moderate ERK1/2 activation as well. Thrombin-stimulated ERK1/2 activation is mainly mediated by PAR-1 via two branches: 1) the PTX-sensitive G proteinβγ-subunit)-phosphatidylinositol 3-kinase branch, and 2) the GqPLC-(InsP3 receptor/ Ca2+-PKC pathway. Thrombin- or PAR-1-AP-induced ERK activation is partially blocked by a selective EGF receptor inhibitor, AG1478. Nevertheless, transphosphorylation of EGF receptor is unlikely for ERK1/2 activation and is certainly not involved in PAR-1-induced proliferation. The metalloprotease mechanism involving transactivation of the EGF receptor by released heparin-binding EGF was excluded. EGF receptor activation was detected by the receptor autophosphorylation site, tyrosine 1068. Our data suggest that thrombin-induced mitogenic action in astrocytes occurs independently of EGF receptor transphosphorylation.

protease-activated receptors; extracellular signal-regulated protein kinase; calcium signaling; epidermal growth factor receptor; transactivation; mitogen-activated protein kinase

IN ADDITION TO BEING A PROTEASE involved in blood coagulation and tissue repair, thrombin has also been shown to act as a multifunctional signaling molecule, even in the brain (19). In astrocytes, thrombin has been found to induce morphological changes, proliferation, and secretion of endothelin-1 (13, 18). These thrombin-stimulated cellular events are mediated through the proteolytic activation of a seven-transmembrane domain G protein-coupled receptor (GPCR) (5, 19), the so-called protease-activated receptor (PAR). Activation of PARs is achieved when the extracellular NH2 terminus of the receptor is cleaved by the specific protease. The newly generated NH2 terminus binds like a tethered ligand intramolecularly to extracellular loop 2 of the receptor (34), leading to the G protein-coupled signal transduction, i.e., activation of phospholipase C (PLC), generation of inositol 1,4,5-trisphosphate (InsP3), increase of intracellular Ca2+, and activation of protein kinase C (PKC). Synthetic peptides mimicking the sequence of the tethered ligand can bind to and activate the receptor, bypassing the requirement of proteolysis. Thrombin can activate PAR-1, -3, and -4 of the PAR family, whereas PAR-2 is mainly activated by trypsin (38).

Activation of mitogen-activated protein kinases (MAPKs) that comprise the extracellular signal-regulated protein kinase ERK1 (p44 MAPK) and ERK2 (p42 MAPK) plays a crucial role in regulating cellular proliferation and differentiation signals from the cell surface to the nucleus (39). The initial characterization of the activation mechanisms of MAPKs by cell surface receptors was revealed by analysis of classic tyrosine kinase receptors such as the epidermal growth factor (EGF) receptor (15). Multiple subsequent studies showed that stimulation of many GPCRs also leads to rapid activation of the ERK pathway (12). Most recently published data suggest that part of the mitogenic stimulus of some GPCRs can be produced by transactivation of EGF receptor (8, 9, 45). The transactivation mechanism was found to be due to release of soluble EGF receptor ligand upon stimulation of GPCRs by thrombin, lysophosphatidic acid (LPA), endothelin, and carbachol (21, 46).

Previous work in our laboratory has shown that all four different types of PARs known so far are widely expressed in the brain (54). We have also demonstrated that rat astrocytes functionally coexpress these four subtypes of PARs (62). Short-term application of agonists for PAR-1 through -4 induces increase in intracellular Ca2+. Furthermore, we found that stimulation of PAR-1 and PAR-2 leads to proliferation of astrocytes.
MAPKs are activated by thrombin, leading to proliferation in various cell types (36, 44, 56). Although thrombin has been shown to activate MAPK in astrocytes (4), the nature of the biochemical link from thrombin receptors to MAPKs in astrocytes remains to be delineated. In the present study we examined whether these PAR-evoked mitogenic signals are transmitted through classic G protein-coupled signaling pathways or cross-communication with EGF receptor. Experiments were performed to identify the relationship between cell proliferation and activation of ERK1/2 by using several pharmacological tools.

The novel mechanism implying activation of EGF receptor indirectly by GPCRs potentially also provides new directions for clinical applications. Thrombin and PARs are targets for possible therapeutic interventions to induce neuroprotection. For possible treatment of neurodegenerative diseases, it is highly important to understand whether the transactivation pathway is connected to PARs in brain cells, because PAR-1 activation appears to be able to promote neuronal survival after ischemia (53) or brain trauma (63). The main finding of this study is that activation of PARs stimulates proliferation of rat cultured astrocytes via the ERK/MAPK pathway. This involves two branches. The first branch goes through PLC-InsP3/Ca2+-PKC and converges with the second pathway, which comes from pertussis toxin (PTX)-sensitive G proteins and phosphatidylinositol (PI) 3-kinase. There is, however, no transphosphorylation of EGF receptor, occurring at autophosphorylation site tyrosine 1068.

MATERIALS AND METHODS

Materials. Human thrombin and EGF were from Sigma (St. Louis, MO). The synthetic thrombin receptor agonist peptide (TRaf; Ala-parafluor-Phe-Arg-Cha-homo-Arg-Tyr-NH2) and rat PAR-2-activating peptide (PAR-2-AP) (SLIGRL, H-Ser-Leu-Ile-Gly-Arg-Leu-NH2) were purchased from Neosystem Laboratory (Strasbourg, France). Human PAR-3-AP (TFRGAP, H-Thr-Paraf-Ile-Gly-Ala-Pro-OH) and rat PAR-4-AP (GYPGKP, H-Gly-Tyr-Pro-Gly-Lys-Phe-OH) were purchased from Bachem (Heidelberg, Germany). U-73343, U-73122, 2-aminoethoxydiphenyl borate (2-APB), PD-98059, AG1478, and wortmannin were purchased from Calbiochem (La Jolla, CA); bisindolylmaleimide (GF-109203X) was from Alexis (San Diego, CA).

Cell cultures. Primary astrocyte-enriched cell cultures were obtained from two newborn rats according to a previously published method (58). All experiments conformed to guidelines from Sachsen-Anhalt on the ethical use of animals, and all efforts were made to minimize the number of animals used. In brief, newborn rats were decapitated, and brains were gently passed through nylon mesh (136-μm pore width) and centrifuged at 500 g for 5 min at 4°C. The brains were resuspended in 10 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 24 h before experiments. All experiments were carried out with a minimum of six wells per condition (n > 6) with at least two different preparations. For assessing the proliferation 24 h later, we used the CellTiter 96 AQ solution. One solution cell proliferation assay (Promega, Madison, WI) in accordance with the manufacturer’s instructions. Absorption was measured at 490 nm with a microplate reader (Molecular Devices). Proliferation is given as the percent change compared with control. The proliferative effect induced by thrombin, TRaf, or PAR-2-AP was further confirmed by measurement of 5-bromodeoxyuridine (BrdU) incorporation according to Yeh et al. (64).

ERK1/2 phosphorylation. Confluent cells were deprived of serum for 24 h before use, and drug treatments were carried out at 37°C as indicated in RESULTS. After stimulation, monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in modified RIPA buffer (50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na2VO4, 1 mM NaF, and one tablet of protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) per 50 ml). The cell lysate was gently shaken on a rocker for 15 min at 4°C. The lysate was centrifuged at 10,000 g for 15 min, the supernatant was immediately transferred to a fresh centrifuge tube, and the pellet was discarded. Protein concentration was determined by the Bradford method using bovine serum albumin as standard. Samples containing equal amounts of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis (20 μg/lane) and transferred to nitrocellulose membrane. Membranes were blocked with...
5% nonfat dry milk for 1 h at room temperature and rinsed in PBS with 0.1% Tween 20 3 times. Membranes were then incubated for 90 min at room temperature with specific antibodies against phosphorylated ERK1/2 [phospho-p44/42 MAPK (Thr202/Tyr204, 1:2000)] or against ERK1/2 [p44/42 MAPK (1:2,000)] (New England Biolabs, Beverly, MA). After three rinses, membranes were further incubated for 90 min at room temperature with peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:10,000, respectively; Dianova, Hamburg, Germany). Membranes were washed three times, and proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). Band intensity was quantified by using a GS-800 calibrated densitometer (Bio-Rad) with Quantity One quantitation software.

**Immunoprecipitation and immunoblotting.** In the experiments for establishing possible EGF receptor activation, stimulations were carried out at 37°C in serum-free medium. After stimulation, monolayers in 60-mm culture dishes were washed twice with ice-cold PBS and lysed in ice-cold modified RIPA buffer. The cell lysate was treated as described in **ERK1/2 phosphorylation**. Protein (500 μg) was incubated with 5 μg of rabbit polyclonal antibody against the EGF receptor (New England Biolabs) for 4 h at 4°C and then with Protein A-conjugated agarose beads overnight with constant shaking at 4°C. Immune complexes were washed three times with ice-cold RIPA buffer, denatured in Laemml sample buffer, and resolved by 7.5% SDS-PAGE. Tyrosine phosphorylation or the presence of immunoprecipitated proteins was detected by protein immunoblotting. Phosphotyrosine was detected by using a 1:500 dilution of anti-phosphotyrosine monoclonal antibody clone 4G10 (Biomol). EGF receptor protein was detected by using a 1:500 dilution of rabbit polyclonal antibody against the EGF receptor.

**Statistics.** Statistical evaluation was carried out using Student’s t-tests, and P<0.05 was considered to be significant. Data are given as means ± SE. All control values are relative values calculated by dividing all single absolute values by the mean of all control values (an absolute value) and then making a group statistic of those relative values.

**RESULTS**

**Activation of PARs stimulates ERK1/2 phosphorylation.** To examine whether stimulation of PARs can elicit ERK1/2 activation in astrocytes, we challenged serum-starved astrocytes with thrombin (1 U/ml) for varying lengths of time, ranging from 5 min to 3 h. The amount of phosphorylated ERK1/2 in astrocytes was determined by Western blot analysis and was normalized by the total amount of ERK1/2. As shown in Fig. 1, A and B, thrombin can time-dependently induce ERK1/2 phosphorylation in astrocytes. The strongest activation was obtained at 5 min, which is consistent with other data (4). This phosphorylation decreased gradually but persisted for up to 3 h. In the following study, the phosphorylation of ERK1/2 is expressed as a percentage of the phosphorylation of ERK1/2 seen after 5 min of stimulation with thrombin (10 U/ml) (see Fig. 2A).

When astrocytes were treated with increasing concentrations of thrombin (0.01–10 U/ml) for 5 min, ERK1/2 were concentration-dependently phosphorylated with the most pronounced effect at the concentration of 10 U/ml, as shown in Fig. 2, A and F. Because thrombin activates not only PAR-1 but also PAR-3 and -4, we had to differentiate between different PARs. Therefore, we also applied the respective PAR-APs as stimulatory agents. As shown in Fig. 2, B–F, the activating peptides for PAR-1, -2, -3, and -4 each evoke some ERK1/2 activation, but to a very different degree. Compared with the response induced by 10 U/ml thrombin, 95% was achieved by 10 μM thrombin receptor agonist (T Rag), which is a potent (16) and specific PAR-1-AP (28). Only 40% activation could be achieved by PAR-2-AP (SLIGRL) at 500 μM. However, a negligible stimulation of 10 and 8% was elicited by PAR-3-AP and PAR-4-AP (500 μM TFRGAP and GYPGKF), respectively. Compared with control cells, significant ERK1/2 phosphorylation was also observed with 0.1 and 1 μM TRag stimulation, 10–100 μM PAR-2 AP stimulation, and 100 μM PAR-3 AP stimulation. PAR-3-AP (TFRGAP) was initially shown to be inactive (26). It is intriguing that we found that this peptide could elicit Ca2+ mobilization in astrocytes (62). In addition, PAR-3-AP is also capable of stimulating ERK1/2, as shown here. Our previous studies had indicated that this peptide is most unlikely to signal through PAR-1 or PAR-2 (62) but, rather, genuinely activates PAR-3. Nevertheless, caution is still needed...
to interpret these results. To clarify unequivocally that PAR-3-AP is indeed activating PAR-3 will require independent expression of rat PAR-3 against a null background and examination of its signaling abilities.

Taken together, these results suggest that activation of PARs stimulates ERK1/2 phosphorylation in astrocytes. However, not all PAR subtypes contribute to the effects induced by thrombin stimulation. The effect of thrombin is mediated mainly through PAR-1, because the effect can almost completely be mimicked by TRag. This is consistent with our recently reported data about proliferation induced by thrombin and TRag in astrocytes (62), as also shown in Fig. 3C. Thus, within the PAR system, PAR-1 seems to be the predominant receptor for the subsequent cellular consequence of exposure of astrocytes to thrombin. Therefore, in the following experiments the elucidation of the transduction mechanism underlying the thrombin response focused on the signaling cascades mediated through PAR-1.

**Role of ERK/MAPK in the mitogenic process initiated by PAR-1 activation.** ERK1/2, which are believed to be a key component for the mitogenic signal transduction, are phosphorylated as a result of PAR-1 activation in a variety of cell types (14, 36, 56). Therefore, PD-98059, a specific MAP kinase kinase (MEK) inhibitor, was applied in the proliferation assays where astrocytes were treated with thrombin or TRag. As shown in Fig. 3C, both thrombin (10 U/ml)- and TRag (10 μM)-induced proliferation in astrocytes were totally blocked by PD-98059 (100 μM). Interestingly, as shown in Fig. 3, A and B, a 15-min preincubation with PD-98059 completely suppressed the ERK1/2 phosphorylation induced by thrombin (1 U/ml) or TRag (1 μM) as well. These results indicate that the proliferation-enhancing effect of thrombin and TRag was mediated through ERK/MAPK activation.

**Effect of PTX on thrombin-induced proliferation and ERK phosphorylation.** PARs are GPCRs signaling via heterotrimeric G proteins. Thus the type of G proteins
involved in thrombin-induced [Ca\(^{2+}\)]\(_i\) mobilization as well as proliferation and ERK1/2 phosphorylation in astrocytes was studied by applying PTX. PTX can inactivate the Gi/Gi family but not affect others. Cells were incubated with PTX (200 ng/ml) for 24 h before stimulation.

Table 1. Inhibition of the [Ca\(^{2+}\)]\(_i\) response induced by PAR-1 activation in rat astrocytes by various agents

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Response, %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Thrombin (0.1 U/ml)</td>
</tr>
<tr>
<td>PTX (200 ng/ml)</td>
<td>56 \pm 4 (94)</td>
</tr>
<tr>
<td>U-73112 (5 (\mu)M)</td>
<td>12 \pm 2 (68)</td>
</tr>
<tr>
<td>U-73343 (5 (\mu)M)</td>
<td>91 \pm 4 (83)</td>
</tr>
<tr>
<td>2-APB (500 (\mu)M)</td>
<td>10 \pm 1 (59)</td>
</tr>
<tr>
<td>Wortmannin (5 (\mu)M)</td>
<td>61 \pm 4 (55)</td>
</tr>
<tr>
<td>GF-109203X (1 (\mu)M)</td>
<td>57 \pm 4 (90)</td>
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Astrocytes were superfused in the absence or presence of U-73343, U-73112, 2-aminoethoxydiphenylborate (2-APB), wortmannin, or GF-109203X at the concentrations indicated for 5 min before the cells were stimulated by protease-activated receptor (PAR)-1 agonist for 1 min. For the treatment with PTX, the cells were preincubated for 24 h before the stimulation. The maximal change in fluorescence ratio (\(\Delta F/F_{\text{ref}}\)) was measured in single cells. Experiments were repeated in at least 2 different preparations, with \(>10\) cells examined for each experiment. The relative response amplitude is given by comparison with the amplitude of Ca\(^{2+}\) response in untreated cells. Data are presented as means \(\pm\) SE, with the number of cells provided in parentheses.

After preincubation with PTX, the increase in [Ca\(^{2+}\)]\(_i\) evoked by thrombin or TRag was attenuated by 44 and 63\%, respectively (Table 1). As Fig. 4C shows, pretreatment with PTX also strongly inhibited the proliferative effects of thrombin and TRag in astrocytes. Moreover, ERK1/2 phosphorylation by thrombin and TRag was also partially diminished due to the Gi/Gi protein inactivation by PTX (Fig. 4, A and B). This partial inhibition was not due to a nonspecific effect because EGF-stimulated ERK1/2 phosphorylation was not affected by the PTX pretreatment (Fig. 4, A and B). These results suggest that the signaling cascade from PAR-1 to the ERK/MAPK is mediated through PTX-sensitive as well as PTX-insensitive G proteins.

Association of InsP\(_3)/Ca\(^{2+}\) with thrombin-induced proliferation and ERK phosphorylation. Activation of PAR-1 results in elevation of intracellular Ca\(^{2+}\) in astrocytes through both Ca\(^{2+}\) release from internal stores and Ca\(^{2+}\) influx (7, 58, 59). Measurements of intracellular Ca\(^{2+}\) in astrocytes showed that the transient rise in [Ca\(^{2+}\)]\(_i\) elicited by short-term stimulation with thrombin and TRag can be nearly completely blocked by application of U-73122 (5 \(\mu\)M), a PLC inhibitor, and 2-APB (500 \(\mu\)M), a noncompetitive antagonist of the intracellular InsP\(_3\) receptor (see data in Table 1). Because activation of PLC and liberation of InsP\(_3\) are events upstream of the Ca\(^{2+}\) release from intracellular stores, our results suggest that the initial Ca\(^{2+}\) response induced by short pulses of thrombin and TRag is mainly caused by Ca\(^{2+}\) release. This rise in [Ca\(^{2+}\)]\(_i\) may also be involved in the subsequent mitogenic signaling cascade induced by PAR-1 activation.

To determine whether PLC and InsP\(_3\) are upstream factors of the proliferative effect, we employed U-73122 and 2-APB in the ERK phosphorylation and proliferation assay. As shown in Fig. 5, pretreatment with U-73122 (5 \(\mu\)M) for 10 min significantly attenuated the proliferative effects of thrombin and TRag in astrocytes. Moreover, ERK1/2 phosphorylation by thrombin and TRag was also partially diminished due to the Gi/Gi protein inactivation by PTX (Fig. 4, A and B). These results suggest that the signaling cascade from PAR-1 to the ERK/MAPK is mediated through PTX-sensitive as well as PTX-insensitive G proteins.

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thrombin- and TRag-induced ERK1/2 phosphorylation.
Interestingly, proliferation in astrocytes was reduced to a similar degree.

U-73343 (5 \( \mu \)M), an inactive analog of U-73122 that is frequently used as a control compound for U-73122, did not affect thrombin- and TRag-mediated \([Ca^{2+}]_i\) increase, ERK1/2 phosphorylation, and proliferation in astrocytes (data in Table 1 and Fig. 5). EGF-induced ERK1/2 activation was not influenced by U-73122 (Fig. 5, A and B). These results confirm the conclusion that the attenuation seen with U-73122 is not a nonspecific inhibition.

Figure 6 shows that 2-APB (500 \( \mu \)M) exerts much stronger inhibitory effects than U-73122 (Fig. 5). Both thrombin- and TRag-induced ERK1/2 phosphorylation and enhancement of proliferation were blocked substantially by treatment with 2-APB. A small inhibition by 2-APB was observed in EGF-evoked ERK phosphorylation, which is negligible when compared with the strong inhibition of thrombin- and TRag-induced responses. In addition, pretreatment with Ca\(^{2+}\) ionophore A-23187 (300 nM) was tested to elucidate the role of Ca\(^{2+}\) in thrombin-induced cell proliferation. A-23187 can induce significant ERK1/2 phosphorylation and proliferation in astrocytes, respectively (Fig. 6), providing further evidence for the contribution of intracellular Ca\(^{2+}\). Taken together, these data demonstrate the involvement of PLC and InsP\(_3\)/Ca\(^{2+}\) in...
thrombin- and TRag-induced mitogenic response in rat astrocytes.

Role of PI 3-kinase within thrombin-induced proliferation and ERK phosphorylation. In the present study we have shown that ERK1/2 phosphorylation in response to thrombin and TRag in astrocytes involves PTX-sensitive G proteins such as G\textsubscript{i}. Some reports suggested that G\textsubscript{i} proteins can activate MAP kinases through their G\textsubscript{i}/G\textsubscript{q} subunits, an effect that was mediated via PI 3-kinase. Therefore, we tested the role of PI 3-kinase in thrombin- and TRag-induced cellular events in astrocytes. As shown in Table 1, treatment of astrocytes with the PI 3-kinase inhibitor wortmannin (5 \(\mu\)M) attenuated thrombin- and TRag-induced Ca\textsuperscript{2+} response by 39 and 63\%, respectively. Results in Fig. 7 demonstrate substantial inhibition by wortmannin of thrombin- and TRag-induced ERK1/2 phosphorylation (by at least 81 and 98\%) and proliferation (by 81 and 83\%), confirming the involvement of PI 3-kinase. In this case, only a small inhibition was observed with EGF-stimulated ERK1/2 phosphorylation by wortmannin.

Involvement of PKC in thrombin-induced proliferation and ERK phosphorylation. Signaling from GPCR to the ERK/MAPK cascade can be transmitted by several distinct pathways, some of which involve PKC. PKC is activated by diacylglycerol, which is generated during the hydrolysis of phosphatidylinositol thrombin- and TRag-induced mitogenic response in rat astrocytes.

![Graph](http://ajpcell.physiology.org/)
4,5-bisphosphate after PLC activation. To examine the role of PKC in thrombin- and TRag-mediated effects in astrocytes, we pretreated cells with the PKC inhibitor GF-109203X (1 μM). Cells treated with GF-109203X showed a decreased Ca²⁺ response to thrombin and TRag stimulation, with 43 and 55% reduction, respectively. Furthermore, biochemical studies showed that thrombin- and TRag-induced ERK1/2 phosphorylation and proliferation were significantly inhibited by pretreatment with GF-109203X, as shown in Fig. 8. ERK1/2 phosphorylation was reduced by 73 and 74%, and proliferation by 59 and 74%. No inhibition was observed with GF-109203X on EGF-stimulated ERK activation, indicating that PKC specifically plays an important role in PAR activation-induced responses in astrocytes.

Question of transactivation of EGF receptor in thrombin-induced proliferation and ERK phosphorylation. Recently emerging evidence has indicated that in certain cell types, the mitogenic effect of thrombin stimulation can be mediated through transactivation of the EGF receptor (9, 27). To verify the nature of the mitogenic actions of thrombin and PAR-1 in astrocytes, we used AG1478 (5 μM), an inhibitor of EGF receptor kinase.

Fig. 9. Effects of AG1478 on ERK1/2 phosphorylation and astrocyte proliferation induced by EGF and PAR-1 activation. Serum-starved astrocytes were preincubated with AG1478 (5 μM) for 15 min before 5 min of stimulation with thrombin (1 U/ml), TRag (1 μM), or EGF (50 ng/ml). A: representative blot of ERK1/2 phosphorylation from 1 experiment. B: quantification by densitometry. Data represent means ± SE of at least 3 experiments. **P < 0.01 vs. cells exposed to EGF alone. C: serum-starved astrocytes were preincubated with AG1478 (5 μM) for 15 min before 3 h of stimulation with thrombin (10 U/ml) or TRag (10 μM). Proliferation is expressed as percent change compared with control. Data represent means ± SE (n = 6 wells/condition). **P < 0.05 vs. cells exposed to thrombin or TRag alone.
First, as shown in Fig. 9, A and B, we found that stimulation of cells for 5 min with EGF also induced the phosphorylation of ERK1/2. According to the density of the phosphorylation signal on the blot, EGF (50 ng/ml; ~0.8 nM) caused a more robust ERK1/2 activation than thrombin (1 U/ml) and TRag (1 μM). The EGF effect can be totally suppressed by pretreatment with AG1478 and the MEK inhibitor PD-98059. While inducing a smaller maximum activation of ERK1/2 than EGF under the experimental conditions, thrombin- and TRag-stimulated ERK1/2 activation was only partially blocked by AG1478. This result, however, is not yet sufficient evidence to clarify the question of whether or not EGF receptor transactivation occurs following PAR-1 activation. Next, AG1478 was tested in the proliferation assay. As shown in Fig. 9C, similar to the inhibition of ERK1/2 phosphorylation by AG1478, EGF induced-proliferation was completely inhibited by pretreatment of AG1478, whereas only a small (statistically insignificant) reduction was observed with thrombin- and TRag-stimulated proliferation. This result suggests the possibility that AG1478 might exert some small, nonspecific inhibition of tyrosine kinase phosphorylation in astrocytes.

Therefore, the concentration dependence of inhibition by AG1478 was measured for EGF- and PAR-1-induced ERK1/2 phosphorylation. As shown in Fig. 10, the potency of AG1478 to inhibit PAR-1-mediated activation of ERK1/2 was almost an order of magnitude greater than its potency to inhibit EGF-induced ERK1/2 activation. However, maximally, AG1478 caused an ~55% inhibition of ERK activation, whereas this compound was able to inhibit EGF-stimulated ERK phosphorylation completely. Given the known specificity of AG1478 for the EGF receptor kinase, this result could be interpreted to indicate that 55% of the ability of PAR-1 to activate ERK1/2 could conceivably involve the EGF receptor, even though autophosphorylation of the receptor cannot be detected (see below). Alternatively, the ability of AG1478 to inhibit PAR-1-stimulated ERK activation by 55% might be due to its inhibition of a kinase distinct from the EGF receptor. Whatever this interesting kinase might be, it is very sensitive to the inhibitor AG1478.

To clearly determine whether the partial inhibitory effects of AG1478 on thrombin- or TRag-induced response were due to the inhibition of EGF receptor activation subsequent to PAR-1 activation, we then examined the phosphorylation status of the EGF receptor. In these experiments the EGF receptor was analyzed by immunoprecipitation analysis. As shown in Fig. 11A, 5 min of stimulation with EGF elicited pronounced tyrosine phosphorylation of the EGF receptor, as shown by probing EGF receptor immunoprecipitates on the blot with phosphotyrosine antibodies. This EGF receptor phosphorylation was totally blocked by pretreatment of astrocytes with AG1478 (5 μM). In contrast, no signal of phosphorylated EGF receptor was observed with either thrombin or TRag stimulation, indicating that EGF receptor has minimal, if any, involvement in PAR-1-induced astrocytic proliferation.

Moreover, to corroborate these results, we have also employed an immunoblotting assay using an antibody that binds to phospho-EGF receptor specifically at tyrosine 1068. Tyrosine 1068 is one of the major sites accounting for EGF receptor autophosphorylation (25). Phospho-tyrosine 1068 of activated EGF receptor is a direct binding site for the Grb2/Sos-1 signaling mechanism (65). Phosphorylation of EGF receptor at tyrosine 1068 has also been implicated under transactivation by GPCR (60). However, in our study, as shown in Fig. 11B, no phosphorylation of the EGF receptor residue tyrosine 1068 was detected after either thrombin or TRag stimulation. Because AG1478 failed to inhibit PAR-1-induced astrocyte proliferation, EGF receptor transactivation did not appear to be involved in the mitogenic action of PAR-1.

Finally, to exclude the possibility that a small degree of tyrosine phosphorylation of EGF receptor that might be below the level detectable by the Western blot would be sufficient for maximal MAPK activation, the correlation between the concentration-effect curve of EGF-stimulated ERK phosphorylation and EGF-mediated EGF receptor tyrosine phosphorylation was examined. As shown in Fig. 11C, 1 ng/ml EGF elicited a detectable signal of phosphorylated EGF receptor together with a level of ERK1/2 phosphorylation that was similar to that induced by stimulation with thrombin (1 U/ml), whereas thrombin stimulation yielded no EGF receptor phosphorylation. The ERK1/2 phosphorylation by 1 ng/ml EGF and by thrombin (1 U/ml) was 65 and 80% of that induced by 50 ng/ml EGF, respectively. Taken together, these results demonstrate that the mitogenic response induced by thrombin and TRag is mediated primarily through the PAR-1-connected signaling pathways, independent of EGF receptor transphosphorylation.
The goal of the present study is to further the understanding of the signal transduction mechanisms underlying thrombin-induced proliferation in astrocytes. Previously, we have shown that rat astrocytes functionally coexpress all four subtypes of PARs. A comparable proliferation was obtained when astrocytes were exposed either for 3 or 24 h to thrombin, TRag, or PAR-2-AP (62). This finding suggests that the initial signaling induced by activation of PAR-1 or PAR-2, especially PAR-1, is sufficient to trigger the proliferation of astrocytes. These results are in line with previous reports that thrombin acts as a mitogen for astrocytes through PAR-1 (18). In other cell types, such as human cultured tracheal smooth muscle cells (36), mouse lung fibroblasts (56), and airway smooth muscle cells (44), it has been clearly shown that MAPKs are activated by thrombin, leading to cell proliferation. Therefore, it was hypothesized that ERK1/2 may also play a central role in the thrombin/PAR-1-evoked proliferative effect in astrocytes.

It was shown in the present study that stimulation by thrombin activated ERK1/2 in astrocytes. Interestingly, we found that the respective PAR-AP acted in a mode similar to the protease to activate ERK1/2, but to a different degree. TRag, a synthetic specific agonist of PAR-1, induced a response resembling in amplitude that of thrombin, not only with ERK1/2 activation (95% of the maximum response inducible by thrombin) but also with intracellular Ca2+ mobilization and astrocytic proliferation (62). However, PAR-2-AP exhibited a smaller potency on ERK1/2 phosphorylation and proliferation. In accordance with our previous data showing that the Ca2+ signal evoked by PAR-3 and PAR-4-AP was relatively weak and that both peptides lack the ability to induce proliferation in astrocytes (62), only a small, almost negligible response was observed on ERK1/2 phosphorylation in this study (Fig. 2F). These results suggest that activation of PARs stimulates ERK1/2 phosphorylation in astrocytes. Furthermore, the data show a close correlation between the amplitude of the Ca2+ response and the extent of ERK1/2 activation as well as proliferation induced by activation of PARs in astrocytes. We have demonstrated that thrombin utilizes PAR-1, -3, and -4 for signal transduction in astrocytes (62), but it seems that PAR-1 is the most prominent receptor among PARs for mediating the cellular consequence of thrombin stimulation in astrocytes.

Moreover, PD-98059, an inhibitor of the ERK activator MEK, was employed to elucidate the relationship of ERK1/2 phosphorylation and proliferation induced by thrombin and TRag. PD-98095 has been shown to block ERK stimulation and to inhibit growth factor-induced proliferation in Swiss 3T3 mouse fibroblasts and rat kidney cells. PD-98095 is highly selective for MEK, as evidenced by its failure to inhibit 18 other serine/threonine protein kinases in vitro and in vivo, including the ERK homolog Jun NH2-terminal kinase (1). The result that PD-98095 abolished effects of both thrombin and TRag in astrocytes supports our hypothesis that proliferation induced by PAR-1 activation is mediated through ERK1/2 activation in astrocytes.

**DISCUSSION**

The goal of the present study is to further the understanding of the signal transduction mechanisms underlying thrombin-induced proliferation in astrocytes. Previously, we have shown that rat astrocytes...
evidence that PTX-sensitive G proteins mediate mitogenic responses and activation of MAPK cascades elicited by a variety of G protein-coupled receptors (24, 61). In cultured rat astrocytes, we found that pretreatment of cells with PTX, which inhibits G<sub>i</sub>/G<sub>q</sub> proteins, partially attenuated all responses induced by thrombin and TRαγ: the Ca<sup>2+</sup> signal, ERK1/2 activation, and proliferation. A similar effect of PTX on thrombin and thrombin receptor-activating peptide (TRAP-14)-induced DNA synthesis has been reported in astrocytes (10). The specificity of PTX and the other inhibitors discussed below was proven in the present study by positive controls for activation of ERK1/2. In addition, results presented in Table 1 also showed positive evidence for the inhibitory activities of PTX, U-73122, and 2-APB on Ca<sup>2+</sup> response induced by PAR-1 activation.

The mechanism of receptor tyrosine kinase (RTK)-stimulated mitogenic signaling involves the formation of complexes between the guanine nucleotide exchange protein Sos and the adaptor protein Grb2 with another tyrosine-phosphorylated adaptor protein, Shc. Recent studies have shown that some GPCRs utilize the same effectors as the RTK pathway (e.g., Shc-Grb-Sos), resulting in Ras and MAPK activation. This cascade is initiated by βγ-subunits and involves a wortmannin-sensitive PI 3-kinase (22, 43). G<sub>i</sub> coupled receptors have been proposed to regulate Ras-dependent signaling cascades through the release of G protein βγ-subunits. We tried to clarify the issue of whether G<sub>βγ</sub> and/or PI 3-kinase are involved in thrombin- and TRαγ-stimulated Ca<sup>2+</sup> signal, ERK1/2 phosphorylation, and proliferation. Therefore, astrocytes were pretreated with wortmannin, the inhibitor of PI 3-kinase. Our results (Fig. 7) showed that the rise in [Ca<sup>2+</sup>]<sub>i</sub> was partially suppressed by wortmannin, whereas ERK1/2 phosphorylation and proliferation were strongly blocked. Obviously, PI 3-kinase plays a decisive role in the signaling pathways initiated by thrombin and TRαγ stimulation in astrocytes. Furthermore, these results provide indirect proof for the possible role of PTX-sensitive G protein βγ-subunits in astrocytes. Such PI 3-kinase-mediated thrombin-induced cell proliferation was also observed in human airway smooth muscle cells (32), aortic smooth muscle cells (51), and human tracheal smooth muscle cells (36). The finding that both RTK and GPCR pathways can activate a similar set of signal transducers indicates more parallels than originally thought.

However, the fact that inhibition by PTX of the increase in [Ca<sup>2+</sup>]<sub>i</sub> and ERK1/2 phosphorylation was only partial in astrocytes indicates the participation also of PTX-insensitive G proteins. In fibroblasts, thrombin initiates the mitogenic signaling pathway by coupling to both PTX-sensitive and -insensitive G proteins (33). PTX-insensitive G proteins like G<sub>α11</sub> give rise to the activation of PLCβ and the generation of InsP<sub>3</sub> and diacylglycerol, which in turn lead to the mobilization of intracellular Ca<sup>2+</sup> and activation of PKC. The data presented in this study have shown that the PLC inhibitor U-73122 substantially suppressed the Ca<sup>2+</sup> mobilization and, to a lesser degree, prevented the ERK1/2 phosphorylation and proliferation induced by thrombin and TRαγ. These downregulation effects were due to the specific inhibition of PLC because U-73343, the inactive analog of U-73122, was ineffective. Meanwhile, the InsP<sub>3</sub> receptor antagonist 2-APB, which has been shown to inhibit InsP<sub>3</sub> receptor-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation in a variety of cell types (37, 55), exerted potent inhibitory effects on thrombin- and TRαγ-stimulated cellular responses as well. Moreover, significant ERK phosphorylation and cell proliferation were also observed with the stimulation by the Ca<sup>2+</sup> ionophore A-23187. These results further demonstrate that PLC and InsP<sub>3</sub>/Ca<sup>2+</sup> act as upstream factors of ERK1/2 phosphorylation in astrocytes, which is in line with ERK1/2 activation induced by endothelin or glutamate in astrocytes (50, 52). In fact, Ca<sup>2+</sup> signaling has been implicated as an important growth signal in many cell types (3, 40). Several Ca<sup>2+</sup>-dependent kinases like proline-rich tyrosine kinase 2 (Pyk2) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) have also been demonstrated to be involved in the MAPK activation pathway in some cell types (41, 48). Whether these Ca<sup>2+</sup>-regulated kinases act as mediators in the PAR-1 signaling cascade as well is currently under investigation.

A number of studies with various GPCRs have demonstrated two signaling pathways from the receptor to the activation of MAPK: a PTX-sensitive, Ras-dependent pathway mediated by G<sub>βγ</sub> and, in addition, a PTX-insensitive, Ras-independent pathway regulated by PKC (11, 23, 29). In the present study, preincubation of astrocytes with PKC inhibitor GF-109203X significantly attenuated the thrombin- and TRαγ-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>, ERK1/2 phosphorylation, and proliferation, implying the essential role of PKC for astrocytic proliferation. In fact, this implication of PKC involvement has been further supported by the inhibitory effects of the PLC inhibitor U-73122 and the InsP<sub>3</sub> receptor antagonist 2-APB, because they are well-established PKC activators. Our previous results have also shown that activation of PKC is required to maintain the refilling of intracellular Ca<sup>2+</sup> stores for sustained thrombin-induced [Ca<sup>2+</sup>]<sub>i</sub> oscillations in rat glioma cells, because addition of GF-109203X irreversibly suppressed thrombin-induced [Ca<sup>2+</sup>]<sub>i</sub> oscillations (57). The inhibitory effects of GF-109203X obtained in this study additionally support the notion that [Ca<sup>2+</sup>]<sub>i</sub> elevation in a variety of cell types

Possible involvement of EGF receptor transactivation in PAR-1-ERK1/2 activation in astrocytes. Despite the fact that activation of GPCRs is able to stimulate mitogenesis in a variety of cell types, several groups have recently implicated the EGF receptor as a necessary signaling component in response to GPCR activation. An alternative mechanism proposed by Daub et al. (9) suggested that GPCRs activate MAPK in Rat-1 fibroblasts through transactivation of the EGF receptor. They further proved that EGF receptor transactivation upon GPCR stimulation involves heparin-bind-
ing EGF-like growth factor and a metalloprotease activity that is rapidly induced upon GPCR-ligand interaction (46). So far, thrombin has been found to cause EGF receptor transactivation in diverse cell types such as HaCaT keratinocytes, COS-7 cells, mouse astrocytes, and rat smooth muscle cells (8, 27).

We speculated that the transactivation mechanism may also account for the PAR activation-induced cellular consequences in rat astrocytes, because initially we found that EGF receptor kinase inhibitor AG1478 partially blocked ERK1/2 phosphorylation induced by thrombin and TRag. However, lack of significant inhibition of thrombin- and TRag-induced proliferation with AG1478 treatment raised the alternative possibility that thrombin and TRag have their own signaling pathways distinct from EGF receptor transactivation. Our comprehensive and detailed experiments trying to detect the phosphorylated EGF receptor further demonstrated that EGF, but not thrombin and TRag, stimulated EGF receptor phosphorylation in rat astrocytes. Interestingly, Crouch et al. (6) very recently showed in Swiss 3T3 cells that thrombin has no direct effect on the activation state of the EGF receptor or of its downstream effectors, although thrombin causes clustering and sensitization of EGF receptor in migrating cells. They showed that DNA synthesis induced by thrombin was resistant to inhibition by AG1478, being only partially inhibited. Similarly, a partial blockade of thrombin-induced ERK1/2 phosphorylation by AG1478 was also observed in their study. AG1478 inhibits the kinase function of EGF receptor by interacting with the ATP binding site in the 1–10 nM range, but its exact mode of inhibition corresponding to the protein substrate is yet unknown (17, 35, 42). Therefore, the partial suppression of thrombin and TRag responses by AG1478 in astrocytes might also be attributed to the inhibition of some unknown kinases. Similarly, in COS-7 cells, PI 3-kinase was confirmed to function as an upstream effector of Ras in GPCR-mediated MAPK stimulation, whereas PI 3-kinase was not involved in cross talk between GPCRs and the EGF receptor. However, an increase in PI 3-kinase activity associated with Grb2 upon LPA treatment was also reversed by AG1478 pretreatment (8).

Metalloproteinases have been proposed as a key intermediate for the release of heparin-binding EGF leading to transactivation of EGF receptor (9, 27). Therefore, we considered this possible involvement and made some experiments with maximastat, an inhibitor of metalloproteinase 9. We did not see any inhibition of thrombin- and TRag-induced ERK phosphorylation by this inhibitor (data not shown). This result supports our interpretation that no EGF receptor phosphorylation could be induced by PAR-1 activation. We still do not know whether EGF receptor transphosphorylation on another tyrosine residue, e.g., Y845, might be involved in the ability of PAR-1 to activate ERK1/2. Although increasing evidence has indicated the cross talk between EGF receptor and GPCRs, transactivation of RTKs does not seem to be a general prerequisite for the activation of MAPK by GPCRs in all cell types, which is evidenced in rat aortic myocytes by 5-hydroxytryptamine stimulation (2), in human epidermoid carcinoma cells by bradykinin stimulation (20), in smooth muscle cells by histamine H1 receptor activation (47), and in human embryonic kidney cells by opioid receptor activation (30).

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**Fig. 12.** Proposed intracellular transduction mechanisms underlying thrombin/PAR-1-induced activation of ERK1/2 and proliferation in astrocytes. Activation of the PAR-1 (7 transmembrane-spanning domains) by thrombin or the selective PAR-1-AP TRag results in both PTX-sensitive and PTX-insensitive G proteins mediating the activation of ERK1/2 cascades leading to proliferation. On one hand, thrombin can activate ERK1/2 via PTX-sensitive G proteins and downstream activation of a tyrosine kinase-dependent process, probably through Ras- and Raf-dependent steps. On the other hand, thrombin activates ERK1/2 via PTX-insensitive G proteins by activation of PLC, resulting in intracellular Ca2+ mobilization and PKC activation, probably through some Ca2+-dependent kinases like Pyk2 leading to subsequent ERK1/2 phosphorylation. PAR-1 and EGF receptor may recruit some common signaling complex leading to Ras activation. However, the EGFR does not seem to participate in PAR-1 signaling in rat astrocytes. DAG, diacyl glycerol; IP3, inositol 1,4,5-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate.
In summary, in this report we have elucidated the mechanism by which thrombin and TRag induce astrocytic proliferation. The pathways established are summarized in the scheme shown in Fig. 12. We have demonstrated that thrombin and TRag induce a mitogenic stimulus via ERK1/2 activation only through G protein-linked signaling, i.e., the PTX-sensitive G protein (βγ subunits)-PI 3-kinase branch and the Gαi-PLC-(InsP3 receptor) Ca2+-PKC pathway but deliver very little or most likely no signal to EGF receptor tyrosine kinase to evoke their mitogenic response. Our results suggest that transactivation of EGFR might contribute only in some cell types to GPCR-mediated mitogenic signaling.

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