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The role of the Ca^{2+}-sensitive tyrosine kinase Pyk2 and Src in thrombin signalling in rat astrocytes

Hong Wang and Georg Reiser

Medizinische Fakultät der Otto-von-Guericke-Universität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany

Abstract

We have recently demonstrated that multiple signalling pathways are involved in thrombin-induced proliferation in rat astrocytes. Thrombin acts by protease-activated receptor-1 (PAR-1) via mitogen-activated protein kinase activity. Signalling includes both G_i/($\beta\gamma$ subunits)–phosphatidylinositol 3-kinase and a G_q-phospholipase C/Ca^{2+}/protein kinase C (PKC) pathway. In the present study, we investigated the possible protein tyrosine kinases which might be involved in thrombin signalling cascades. We found that, in astrocytes, thrombin can evoke phosphorylation of proline-rich tyrosine kinase (Pyk2) via PAR-1. This process is dependent on the increase in intracellular Ca^{2+} and PKC activity. Moreover, in response to thrombin stimulation Pyk2 formed a complex with Src tyrosine kinase and adapter protein growth factor receptor-bound protein 2 (Grb2), which could be coprecipitated. Furthermore, both thrombin-induced Pyk2 phosphorylation and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation can be attenuated by Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine. From these data we conclude that PAR-1 uses Ca^{2+}- and PKC-dependent Pyk2 to activate Src, thereby leading to ERK1/2 activation, which predominantly recruits Grb2 in rat astrocytes.

Keywords: astrocytes, calcium, mitogen-activated protein kinase, protease-activated receptor, Pyk2, thrombin.
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Thrombin has been proven to act as a signalling molecule in astrocytes, inducing reversal of stellation, proliferation, synthesis and secretion of nerve growth factor and endothelin-1 (Ehrenreich *et al*. 1993; Neveu *et al*. 1993; Grabham and Cunningham 1995), events that are associated with brain injury. These effects of thrombin are mediated via a cell surface G protein-coupled receptor (GPCR)–protease-activated receptor (PAR) (Coughlin 1994). Thrombin activates its target receptor by cleaving the amino terminus of the receptor. A new amino terminus is exposed and binds as a tethered ligand to the body of the receptor to effect transmembrane signalling. In a similar way, PAR can also be activated by synthetic peptides which comprise the sequences of the tethered ligand. So far, four subtypes of PARs have been identified (PAR-1, -2, -3 and -4). Previously, we have demonstrated that cultured rat astrocytes functionally coexpress the four subtypes of PARs. Although thrombin can activate PAR-1, -3 and -4 in astrocytes (Wang *et al*. 2002a), PAR-1 is the predominant receptor for regulating thrombin-induced proliferation (Wang *et al*. 2002b).

Pathological development or traumatic injury in the CNS may stimulate astrocyte proliferation as part of the repair process and contribute to astrogliosis, which has been implicated in brain injury and also in neurodegenerative diseases, such as Alzheimer's disease (Griffin *et al*. 1989; Vaughan *et al*. 1994). High concentrations of thrombin have often been observed in the brain after breakdown of the blood–brain barrier, thereby possibly causing astrogliosis. We made attempts to characterize the signalling pathway underlying thrombin-induced proliferation in astrocytes in

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Address correspondence and reprint requests to Prof. Georg Reiser, Otto-von-Guericke-Universität Magdeburg, Medizinische Fakultät, Institut für Neurobiochemie, Leipziger Str. 44, 39120 Magdeburg, Germany. E-mail: georg.reiser@medizin.uni-magdeburg.de

Abbreviations used: 2-APB, 2-aminoethoxydiphenylborate; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; Grb2, growth factor receptor-bound protein 2; GST, glutathione transferase; $InsP_3$, inositol 1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PAR, protease-activated receptor; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; PLC, phospholipase C; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-d]pyrimidine; Pyk2, proline-rich tyrosine kinase 2; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; Shc, adapter protein in the Ras (small GTPase) pathway; Sos, son of sevenless, Ras guanine nucleotide-releasing factor; TRag, thrombin receptor agonist.

our recent publication (Wang *et al*. 2002b) as reviewed in Hollenberg (2002).

Investigating thrombin-induced signalling in astrocytes, we demonstrated that thrombin/PAR-1 delivers a mitogenic stimulus via extracellular signal-regulated kinase (ERK) 1/2 activation, which involves two pathways: firstly, a pertussis toxin-sensitive G protein-mediated phosphatidylinositol 3-kinase pathway and, secondly, a pertussis toxin-insensitive G protein-mediated phospholipase C (PLC)/Ca^{2+}/protein kinase C (PKC) pathway. This signalling cascade, however, does not cross-talk with epidermal growth factor receptor, although accumulating data had suggested that part of the mitogenic stimulus of some GPCR agonists, such as thrombin, lysophosphatidic acid, endothelin and carbachol (Prenzel *et al*. 1999; Gschwind *et al*. 2001), can be produced by transactivation of epidermal growth factor receptor (Carpenter 1999; Daub *et al*. 1996; Daub *et al*. 1997; Pierce *et al*. 2001).

Proline-rich tyrosine kinase (Pyk2), also known as cell adhesion kinase β, calcium-dependent tyrosine kinase and related adhesion focal tyrosine kinase, is a novel non-receptor tyrosine kinase with a high sequence homology to p125 focal adhesion kinase. Similar to focal adhesion kinase, Pyk2 has been shown to link GPCRs to ERK1/2 activation by recruiting the intermediates such as growth factor receptor-bound protein 2 (Grb2), adapter protein in the Ras (small GTPase) pathway (Shc) and the nucleotide exchange factor Sos (son of sevenless; Ras guanine nucleotide-releasing factor) (Lev *et al*. 1995). Moreover, Pyk2 seems to operate in concert with c-Src (Dikic *et al*. 1996; Della Rocca *et al*. 1997).

Pyk2 has attracted a great deal of attention because of its sensitivity to increased intracellular free Ca^{2+} levels (Avraham *et al*. 1995; Lev *et al*. 1995; Sasaki *et al*. 1995). While usually mainly distributed diffusely throughout the cytoplasm, Pyk2 is readily activated by various stimuli, including growth factors, neurotransmitters, adhesion to the extracellular matrix and stress signals (Tokiwa *et al*. 1996; Cazaubon *et al*. 1997; Felsch *et al*. 1998; Litvak *et al*. 2000), which increase intracellular calcium concentration and/or activate PKC. As Ca^{2+} is known to be an important intracellular messenger to control cellular processes as diverse as cell proliferation, neuronal plasticity or cell death (Berridge *et al*. 1998), Pyk2 has the potential to play a significant role in the transmission and regulation of cellular signals.

It has been described in some cell types that Pyk2 phosphorylation depends both on the increase in intracellular Ca^{2+} and activation of PKC (Lev *et al*. 1995; Schaller and Sasaki 1997; Sabri *et al*. 1998; Wu *et al*. 2002). In human endothelial cells, however, thrombin-stimulated Pyk2 phosphorylation is dependent on intracellular Ca^{2+} but independent of PKC and Src kinases (Keogh *et al*. 2002). Ca^{2+} and PKC have been implicated in our previous study as playing an important role in regulating thrombin-induced mitogenesis via ERK1/2 pathway (Wang *et al*. 2002b). However, it is still unknown whether Pyk2, the important calcium-dependent tyrosine kinase, might also be involved in thrombin signalling cascades in astrocytes. In the present study, we examined this possibility and came to the conclusion that thrombin can indeed induce Pyk2 activation in astrocytes in a Ca^{2+}- and PKC-dependent manner, which is associated with Src and Grb2, leading to ERK1/2 phosphorylation.

Materials and methods

Materials

Human thrombin and protein G-agarose were from Sigma (St Louis, MO, USA). The synthetic thrombin receptor agonist peptide (TRag, Ala-parafluorPhe-Arg-Cha-homoArg-Tyr-NH_2) was purchased from Neosystem Laboratoire (Strasbourg, France). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) was from Tocris (Ellisville, MO, USA). U73122, 2-aminoethoxydiphenylborate (2-APB) and phorbol-12,13-dibutyrate (PDBu) were purchased from Calbiochem (La Jolla, CA, USA) and bisindolylmaleimide (GF-109203X) was from LC Laboratories (Grünberg, Germany). Anti-Pyk2/cell adhesion kinase β monoclonal antibody (clone 74), anti-phosphotyrosine monoclonal antibody (clone 4G10), anti-Shc polyclonal antibody, anti-Src monoclonal antibody (clone GD11) and agarose-conjugated glutathione-*S*-transferase (GST)-Grb2 fusion protein were from Upstate Biotechnology (Lake Placid, NY, USA). Anti-phospho-Src (Tyr418) polyclonal antibody, anti-phospho-Pyk2 (Tyr402) polyclonal antibody and anti-phospho-Pyk2 (Tyr881) polyclonal antibody were from Biosource International, Camarillo, CA, USA. Anti-phospho-p44/42 mitogen-activated protein kinase (MAPK; Thr202/Tyr204) E10 monoclonal antibody and anti-p44/42 MAPK antibody were from New England Biolabs, Beverly, MA, USA. Horseradish peroxidase-conjugated secondary antibodies were from Dianova (Hamburg, Germany).

Cell cultures

Primary astrocyte cell cultures were obtained from two new-born rats according to a previously published method (Ubl and Reiser 1997). All experiments conformed to guidelines from Sachsen-Anhalt, Germany on the ethical use of animals and all efforts were made to minimize the number of animals used. In brief, new-born rats were decapitated, total brains were removed and collected in ice-cold Puck's-D1 solution composed of (mM): NaCl, 137.0; KCl, 5.4; KH_2PO_4, 0.2; Na_2HPO_4, 0.17; glucose, 5.0 and sucrose, 58.4, pH 7.4. The brains were gently passed through nylon mesh (136 μm pore width) and centrifuged at 4°C for 5 min at 500 *g*. The cells were resuspended in 10 mL Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 20 U/mL penicillin and 20 μg/mL streptomycin (Biochrom, Berlin, Germany). The cells were plated on round coverslips (22 mm diameter) placed in culture dishes (50 mm diameter) at a density of $2.5–5.0 \times 10^5$ cells/dish and incubated at 37°C with 10% CO_2, humidified to saturation. The medium was changed for the first time after 5 days and thereafter every 2–3 days, depending on the cell density. For experiments, cells were used between days 7 and 14 in culture. The purity of astrocyte culture was determined by immunofluorescence using a

mouse monoclonal antibody against glial fibrillary acidic protein (Boehringer, Mannheim, Germany), an astrocyte-specific marker. Alexa™ 488 anti-mouse IgG antibody (Molecular Probes, Eugene, OR, USA) was used as the secondary antibody. Confluent monolayers of astrocytes showed >97% positive staining for glial fibrillary acidic protein.

ERK1/2 phosphorylation

Confluent cells were deprived of serum for 24 h before use and drug treatments carried out at 37°C as indicated in Results. After stimulation, monolayers were washed twice with ice-cold phosphate-buffered saline and lysed in modified RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 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 14 000 *g* in a precooled centrifuge for 15 min and the supernatant fluid was immediately transferred to a fresh centrifuge tube, discarding the pellet. Protein concentration was determined by the Bradford method using bovine serum albumin as standard. Samples containing equal amounts of protein were subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; 20 μg/lane) and transferred to nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk for 1 h at room temperature (20°C) and rinsed three times in phosphate-buffered saline with 0.1% Tween 20. Then followed an incubation 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 : 2000)]. 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). Membranes were washed three times and proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Immunoprecipitation

For immunoprecipitation, the cell lysate (500–1000 μg protein) was incubated with the antibodies for 4 h at 4°C and then with protein G-conjugated agarose beads overnight with constant shaking at 4°C. Immune complexes were washed three times with ice-cold RIPA buffer, denatured in Laemmli sample buffer and resolved by SDS–PAGE. Tyrosine phosphorylation or the presence of immunoprecipitated proteins was detected by protein immunoblotting as described. For immunoblot analysis of Grb2-associable proteins, agarose-conjugated GST-Grb2 fusion protein was mixed with constant shaking with cell lysate at 4°C for 16 h and washed with lysis buffer. Bound proteins were solubilized, resolved by SDS–PAGE and subjected to immunoblotting. When quantification became necessary, band intensity was quantified using a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) with Quantity One Quantification Software.

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 ± SEM.

Results

Thrombin induces Pyk2 phosphorylation in astrocytes through PAR-1

We have reported that thrombin can time-dependently induce ERK1/2 phosphorylation in astrocytes with the maximal activation obtained at 5 min. This phosphorylation decreased gradually but persisted for up to 3 h (Wang *et al*. 2002b). Pyk2 is assumed to be a factor upstream of ERK phosphorylation. To assess whether thrombin activates Pyk2 in addition to eliciting ERK1/2 phosphorylation, serum-starved astrocytes were stimulated with thrombin (1 U/mL) for varying lengths of time (5 min−2 h) followed by immunoprecipitation with anti-Pyk2 and immunoblotting with anti-pTyr antibody. Our results demonstrate that Pyk2 is expressed in primary astrocytes, which is in accordance with other studies (Cazaubon *et al*. 1997; Bajetto *et al*. 2001). Furthermore, thrombin treatment caused a rapid and sustained increase in phosphorylation of Pyk2. The most pronounced effect was obtained at 5 min. The phosphorylation signal remained obvious for at least 120 min (Fig. 1a) which is similar to the time course of the ERK1/2 activation stimulated by thrombin (Wang *et al*. 2002b), implying that Pyk2 may play an important role in the ERK/MAPK cascade.

To verify that activation of Pyk2 by thrombin was receptor-dependent and to determine which thrombin receptor subtype mediated this stimulation, we applied TRag, a potent (Feng *et al*. 1995) and specific PAR-1-activating peptide (Kawabata *et al*. 1999), in our experiments. Consistent with our previous results, TRag induced Pyk2

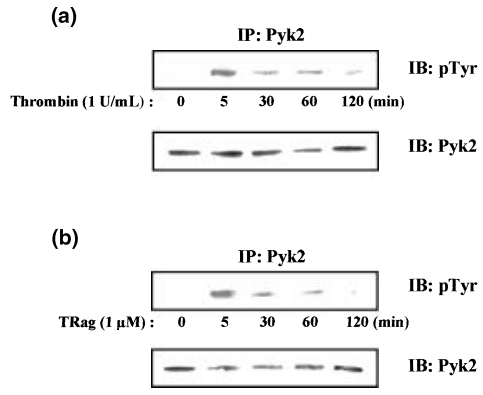

Fig. 1 Time course of proline-rich tyrosine kinase 2 (Pyk2) activation by thrombin in rat astrocytes. Serum-starved astrocytes were exposed to (a) thrombin (1 U/mL) or (b) thrombin receptor agonist (TRag; 1 μM) for the time periods indicated. Phosphorylated Pyk2 was detected by immunoblotting (IB) with anti-phosphotyrosine monoclonal antibody, following immunoprecipitation (IP) with anti-Pyk2. Upper panel, phosphorylation of Pyk2; lower panel, equal loading of protein by detecting total Pyk2. The blots shown are representative of three separate experiments.

phosphorylation in a similar pattern to thrombin (Fig. 1b). The involvement of PAR-3 and PAR-4 can be excluded because no Pyk2 phosphorylation signal could be observed with applications of specific peptides (500 μM) activating either PAR-3 or PAR-4 (data not shown). These results indicate that thrombin activates Pyk2 in astrocytes through PAR-1.

Ca^{2+}-dependent Pyk2 activation in response to PAR-1 activation in astrocytes

We have demonstrated that thrombin and TRag stimulate proliferation in astrocytes via the ERK1/2 pathway in a Ca^{2+}- and PKC-dependent manner. In our previous study we investigated blockade of the upstream factors of Ca^{2+} release from internal stores, such as PLC and inositol 1,4,5-trisphosphate ($InsP_3$) receptor and blockade of PKC activation. Both the inhibition of PKC and of Ca^{2+} release have been shown to attenuate proliferation and ERK1/2 phosphorylation induced by thrombin and TRag (Wang *et al*. 2002b).

In the present study, to clarify the role of Ca^{2+} in thrombin-induced Pyk2 phosphorylation, we applied the intracellular calcium chelator BAPTA-AM, the specific PLC inhibitor U73122 and the $InsP_3$ receptor antagonist 2-APB. We have previously shown that short-term stimulation with thrombin and TRag induced a rapid increase in intracellular Ca^{2+} in astrocytes (Ubl and Reiser 1997). This effect can be inhibited almost completely by U73122 and 2-APB (Wang *et al*. 2002b). Similarly, in the present study we observed that pre-treatment with BAPTA-AM caused a 90% reduction in Ca^{2+} response induced by thrombin and TRag (data not shown). These substances were also employed in the current assay. As presented in Fig. 2(a and b), pre-incubation with BAPTA-AM or 2-APB substantially blocked the Pyk2 phosphorylation stimulated by thrombin and TRag. In addition, Pyk2 phosphorylation in response to thrombin and TRag was also inhibited by U73122 (Fig. 2c). The requirement for Ca^{2+} to activate Pyk2 in astrocytes was further confirmed by assessing Pyk2 phosphorylation following short-term stimulation with Ca^{2+} ionophore A23187, as demonstrated in Fig. 2(a). These results proved the Ca^{2+} dependence for Pyk2 phosphorylation by PAR-1 activation.

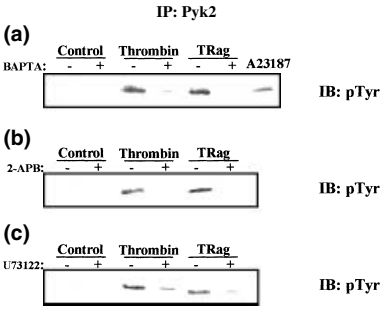

Fig. 2 Involvement of Ca^{2+} in thrombin- and thrombin receptor agonist (TRag)-evoked proline-rich tyrosine kinase 2 (Pyk2) phosphorylation in rat astrocytes. Serum-starved astrocytes were pre-incubated with (a) BAPTA-AM (50 μM), (b) 2-aminoethoxydiphenylborate (2-APB; 100 μM) or (c) U73122 (5 μM) for 10 min prior to 5 min stimulation with thrombin (1 U/mL) or TRag (1 μM). A 5-min exposure to A23187 (2 μM) was also tested (a). Phosphorylated Pyk2 was detected by immunoblotting (IB) with anti-phosphotyrosine monoclonal antibody following immunoprecipitation (IP) with anti-Pyk2. The blots shown are representative of at least three separate experiments.

PKC-dependent Pyk2 activation in response to PAR-1 activation in astrocytes

The requirement of PKC activation in thrombin-induced Pyk2 phosphorylation was examined by pre-treatment with the PKC inhibitor GF109203X. The data presented in Fig. 3(a) show that GF109203X partially diminished thrombin- and TRag-induced Pyk2 phosphorylation. In that blot the differences were not as pronounced as the effect of 2-APB. Therefore, the density of the signal was quantified. The result of the statistical analysis shown in Fig. 3(b) demonstrates a significant difference between Pyk2 phosphorylation in GF109203X-treated and untreated cells, indicating that PKC participates in the regulation of Pyk2 activation. Furthermore, short-term stimulation by PKC

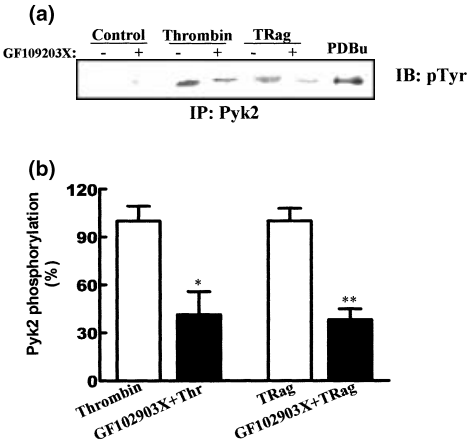

Fig. 3 Involvement of protein kinase C activation in thrombin- and thrombin receptor agonist (TRag)-evoked proline-rich tyrosine kinase 2 (Pyk2) phosphorylation in rat astrocytes. Serum-starved astrocytes were pre-incubated with GF109203X (1 μM) for 10 min prior to 5 min stimulation with thrombin (1 U/mL) or TRag (1 μM). A 10-min exposure to phorbol-12,13-dibutyrate (PDBu; 100 nM) was also tested. Phosphorylated Pyk2 was detected by immunoblotting (IB) with anti-phosphotyrosine monoclonal antibody following immunoprecipitation (IP) with anti-Pyk2. A representative blot from at least three separate experiments is shown in (a). In addition, bands were quantified by a densitometer. The amount of Pyk2 phosphorylation was normalized by referring to the total amount of Pyk2. Data shown in (b) represent the mean ± SEM of at least three experiments. $*p < 0.05$, $**p < 0.01$ versus the value of cells stimulated by protease-activated receptor (PAR)-1 agonist in the absence of GF109203X.

activator phorbol-12,13-dibutyrate (PDBu) also increased Pyk2 phosphorylation, as shown in Fig. 3(a). Thus, our results are consistent with data obtained in other cell types showing that Pyk2 is activated by GPCRs through transient elevation in the cytoplasmic concentration of Ca^{2+} and/or activation of PKC (Lev *et al*. 1995; Sabri *et al*. 1998; Wu *et al*. 2002).

Thrombin stimulates the association of Pyk2 with Src and Grb2

In neuronal cells Pyk2 has been implicated in the mediation of calcium ionophore- and phorbol ester-stimulated ERK1/2 phosphorylation via a direct interaction with c-Src (Dikic *et al*. 1996). To elucidate whether, in astrocytes, ERK1/2 activation induced by stimulation with thrombin or TRag is mediated through Src, firstly, astrocytes were pre-treated with the selective inhibitor of the Src kinase family, PP2. At 10 μM, PP2 significantly abrogated both thrombin- and TRag-induced ERK1/2 phosphorylation (Fig. 4a), suggesting the involvement of Src in the PAR-1/ERK signalling cascades. Secondly, the activation of Src was detected by using a specific antibody which recognizes phospho-Src at Tyr418, an auto-phosphorylation site located in the kinase activation loop (Chiu *et al*. 2002). Full catalytic activity of c-Src kinase requires phosphorylation of Tyr418. As presented in Fig. 4(b), phosphorylation of Src at Tyr418 can be

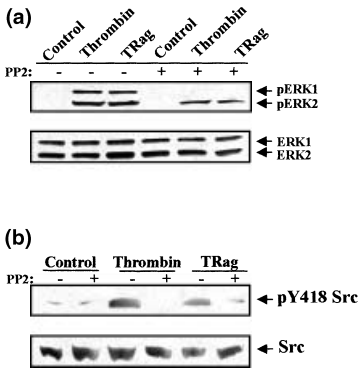

Fig. 4 Src activation induced by thrombin and thrombin receptor agonist (TRag) stimulation in rat astrocytes. Serum-starved astrocytes were pre-incubated with PP2 (10 μM) for 30 min prior to 5 min stimulation with thrombin (1 U/mL) or TRag (1 μM). (a) The amount of phosphorylated extracellular signal-regulated kinase (ERK)1/2 in astrocytes was determined by western blot analysis with antibodies against either phospho-p44/42 mitogen-activated protein kinase (MAPK; Thr202/Tyr204) or p44/42 MAPK. Upper panel, phosphorylation of ERK1/2 induced by protease-activated receptor (PAR)-1 activation; lower panel, equal loading of protein by detecting total ERK1/2. (b) The activation of Src in astrocytes was determined by western blot analysis with antibody against phospho-Src (Tyr^{418}). Upper panel, phosphorylation of Src (Tyr^{418}); lower panel, equal loading of protein by detecting total Src. The blots shown are representative of at least three separate experiments.

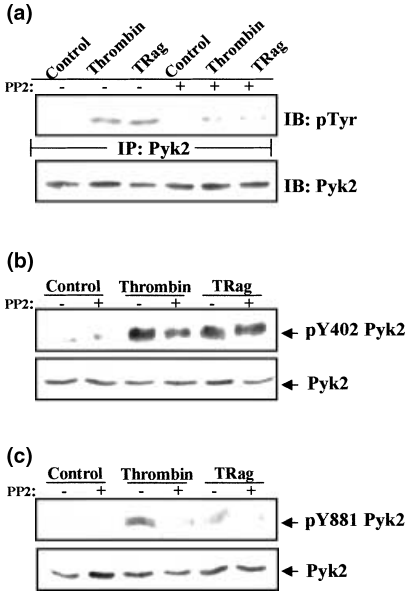

Fig. 5 The influence of Src kinase inhibitor PP2 on proline-rich tyrosine kinase 2 (Pyk2) phosphorylation induced by thrombin and thrombin receptor agonist (TRag) stimulation in rat astrocytes. Serum-starved astrocytes were pre-incubated with PP2 (10 μM) for 30 min prior to 5 min stimulation with thrombin (1 U/mL) or TRag (1 μM). (a) Phosphorylated Pyk2 was detected by immunoblotting (IB) with anti-phosphotyrosine monoclonal antibody following immunoprecipitation (IP) with anti-Pyk2. (b) The phosphorylation of Pyk2 in astrocytes was determined by western blot analysis with specific antibody against phospho-Pyk2 (Tyr^{402}). (c) The phosphorylation of Pyk2 in astrocytes was determined by western blot analysis with second antibody against phospho-Pyk2 (Tyr^{881}). All upper panels, phosphorylation of Pyk2; all lower panels, equal loading of protein by detecting total Pyk2. The blots shown are representative of at least three separate experiments.

induced by 5 min stimulation with thrombin or TRag. This effect is inhibited by pre-incubation with PP2 as well, providing direct evidence that Src is activated by PAR-1 activation and plays a role in thrombin signalling in astrocytes.

Interestingly, as shown in Fig. 5(a), we also observed an inhibition of thrombin- and TRag-induced Pyk2 phosphorylation by PP2, indicating that Src may interact concomitantly with Pyk2 upstream of ERK1/2 activation. It has been documented that the autophosphorylation of Pyk2 at Tyr402 creates a direct binding site for the SH2 domain of Src, leading to Src activation. These steps subsequently enable Src to phosphorylate Pyk2 within the carboxyl terminus at Tyr881 which promotes Grb2 binding (Blaukat *et al*. 1999). It is likely that the blockade of Src kinase by PP2 prevents the phosphorylation of Pyk2 at Tyr881. To further clarify this issue, we applied antibodies which specifically recognize phospho-Pyk2 at either Tyr402 or Tyr881 in western blotting. Results shown in Fig. 5(b) indicate that Pyk2 autophosphorylation at Tyr402 was not affected by PP2 pre-incubation. However, Pyk2 phosphorylation at Tyr881 was dramatically

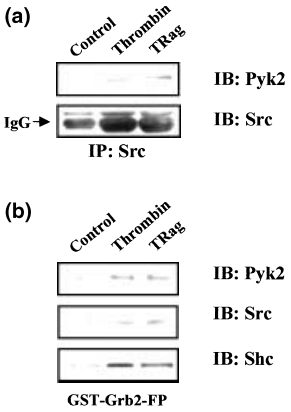

Fig. 6 Thrombin stimulates complex formation of proline-rich tyrosine kinase 2 (Pyk2)-Src-growth factor receptor-bound protein (Grb)2 in rat astrocytes. Serum-starved astrocytes were stimulated with thrombin (1 U/mL) or thrombin receptor agonist (TRag; 1 μM) for 5 min. (a) Lysates were immunoprecipitated (IP) with anti-Src antibody. Immunoblot analysis was performed with anti-Pyk2 (upper panel) and anti-Src (lower panel) antibodies. (b) Lysates were precipitated with glutathione transferase (GST)-Grb2 fusion protein (GST-Gbr2-FP). The precipitates were subjected to immunoblotting (IB) with anti-Pyk2, anti-Src and Shc antibodies.

attenuated by PP2, suggesting that Src can also enhance Pyk2 kinase activity.

The results above strongly imply that a signalling complex is formed following PAR-1 activation, i.e. Pyk2/Src/Grb2, which subsequently leads to ERK1/2 activation. In fact, Fig. 6(a) shows that, after thrombin and TRag stimulation, Pyk2 was detectable by immunoblotting with anti-Pyk2 in the immunoprecipitate of Src. To further examine this hypothesis, we performed coprecipitation experiments with a GST-Grb2 fusion protein followed by immunoblotting with antibodies against either Pyk2 or Src. As shown in Fig. 6(b), treatment with thrombin and TRag induced association of Grb2 with both Pyk2 and Src. These results indicate that PAR-1 activation promotes the interaction of Src with Pyk2. These data provide evidence that activated Pyk2 may recruit the adapter proteins through Src and Grb2 for the subsequent signal transduction.

Besides the Pyk2/Src/Grb2 signalling complex formed following PAR-1 activation, we have also examined the possible interaction between Pyk2 and Shc. It has been reported that thrombin and the thrombin receptor peptide agonist stimulate a rapid and sustained tyrosine phosphorylation of Shc, as well as the formation of Shc-Grb2 complex in CCL39 cells (Chen *et al*. 1996). In our study, we found that the interaction between Grb2 and Shc was significantly enhanced after PAR-1 activation (Fig. 6b). However, no clear signal could be detected by using anti-Shc antibody in the immunoprecipitate of either Src or Pyk2 from cells which were stimulated with thrombin or TRag (data not shown). This indicates that there is no strong direct association between Shc and Src or Pyk2.

Discussion

Previous studies from our laboratory indicated that thrombin induces proliferation in rat astrocytes through an ERK1/2 activation pathway which might be regulated by a Ca^{2+}-dependent tyrosine kinase or kinases. Subsequently, we excluded the possibility that the epidermal growth factor receptor acts as such a tyrosine kinase (Wang *et al*. 2002b). Here, we further investigated and characterized a Ca^{2+}-dependent tyrosine kinase, Pyk2, which is responsive to thrombin stimulation in rat astrocytes.

Pyk2 has been reported to be expressed at high levels mainly in cells of neuronal original (Avraham *et al*. 1995; Sasaki *et al*. 1995). In the present study we provide evidence that, in astrocytes, Pyk2 can be activated by thrombin stimulation. TRag, a synthetic specific agonist of PAR-1, induced a response resembling that of thrombin. However, no such Pyk2 phosphorylation can be induced by peptides activating PAR-3 or PAR-4. Along with previous observations reporting that thrombin, through PAR-1 receptor, acts as a signalling molecule for astrocytes to evoke intracellular Ca^{2+} mobilization (Ubl and Reiser 1997; Ubl *et al*. 2000), ERK1/2 phosphorylation and proliferation (Wang *et al*. 2002b), these results indicate that PAR-1 is the predominant receptor mediating the cellular consequence of thrombin stimulation in astrocytes.

Ca^{2+} signalling has been implicated as an important growth signal in many cell types (Berridge *et al*. 1998; Martinez-Salgado *et al*. 2000). In rat astrocytes an increase in intracellular Ca^{2+} has been demonstrated to play an essential role in transducing the mitogenic signal from PAR-1 receptor to ERK1/2 activation (Wang *et al*. 2002b). The results obtained from the present study suggest that both Ca^{2+}- and PKC-dependent pathways mediate thrombin-induced phosphorylation of Pyk2. The Ca^{2+}-dependence is demonstrated by several lines of experiment. Firstly, Pyk2 was rapidly phosphorylated in response to the Ca^{2+} ionophore, A23187. Secondly, chelation of cytosolic Ca^{2+} by pre-treatment with BAPTA-AM resulted in a significant inhibition of thrombin-induced Pyk2 phosphorylation. Thirdly, Pyk2 phosphorylation was diminished by inhibition of PLC and antagonism of $InsP_3$ receptor which both block Ca^{2+} release from internal pools evoked by PAR-1 activation. In summary, these data are consistent with the involvement of Ca^{2+} in thrombin-induced ERK1/2 phosphorylation in astrocytes, implying that Pyk2 acts as a linker between the Ca^{2+} signal and ERK1/2 activation.

Pyk2 lacks a calmodulin-binding motif. Therefore, it cannot be activated either by Ca^{2+} or calmodulin *in vitro* (Huckle *et al*. 1992; Earp *et al*. 1995; Wilm *et al*. 1996) and the mechanism by which the Ca^{2+} signal activates

Pyk2 remains to be defined. In addition to the Ca^{2+}-dependence, thrombin-induced Pyk2 phosphorylation is also regulated by PKC in astrocytes, which is evidenced by the attenuation of Pyk2 phosphorylation by the selective PKC inhibitor GF109203X and by direct stimulation by PKC activator PDBu. This finding contrasts with some reports of PKC-independent Pyk2 activation in several cell types, including cardiac fibroblast (Murasawa *et al*. 1998) and human endothelial cells (Keogh *et al*. 2002), but it is in accordance with other studies showing a requirement for both PKC and Ca^{2+} (Lev *et al*. 1995; Sabri *et al*. 1998; Wu *et al*. 2002). It seems that, following PAR-1 activation, the Ca^{2+} and PKC pathways converge at the point of Pyk2 in rat astrocytes.

Since initial work on Pyk2 identified this protein as a link between GPCRs and MAPK activation (Lev *et al*. 1995), it is tempting to speculate that Pyk2 activation may be involved in the thrombin-elicited ERK/MAPK pathway. Pyk2 may interact with another tyrosine kinase, Src, and adapter proteins like Grb2 for the subsequent ERK1/2 phosphorylation. Although Pyk2 lacks Src homology 2 and 3 domains (SH2 and SH3), it is known that the auto-phosphorylation of Pyk2 at Tyr402 creates a potential high-affinity binding site for the SH2 domain of Src, leading to Src activation and the formation of a Pyk2-Src signalling complex. Recruitment and activation of Src can further lead to tyrosine phosphorylation of Pyk2 at Tyr 881 to create a binding site for the SH2 domain of Grb2 (Blaukat *et al*. 1999). In addition, Src may also tyrosine phosphorylate the adapter molecule Shc, resulting in its interaction with Grb2 (Yamaguchi *et al*. 1997). Grb2 can then recruit the guanine nucleotide exchange factor Sos, leading to Ras activation and, ultimately, activation of ERK1/2 (Rocic *et al*. 2001). It has been shown that dominant negative mutants of Pyk2 blocked the formation of the Shc-Grb2 signalling complex and, consequently, MAPK activation in PC12 cells (Lev *et al*. 1995; Dikic *et al*. 1996). In the present study, we have demonstrated that Pyk2 forms a complex with both Src and Grb2 in astrocytes in response to thrombin stimulation. In addition, Shc also binds to Grb2 to form another signalling complex for signal transduction. Inhibition of Src tyrosine kinase reduces both Pyk2 phosphorylation at Tyr881 and ERK1/2 phosphorylation following PAR-1 activation, implicating that Pyk2 and Src are intermediates upstream of ERK1/2 activation in astrocytes.

The proposed involvement of Pyk2 in signal transduction of thrombin-induced ERK activation is illustrated in Fig. 7. Ca^{2+} mobilization and PKC activation induced by PAR-1 activation result in phosphorylation of Pyk2 at Tyr402 which, in turn, creates a binding site for Src tyrosine kinase. Binding of Src to phosphorylated Tyr402 leads to Src activation, which is critical for phosphorylation of Pyk2 at Tyr881, and subsequent binding of adapter protein Grb2. A signalling complex, i.e. Pyk2/Src/Grb2, is then formed. This complex, which also interacts through Grb2 with Shc and Sos, transmits signals downstream of Pyk2 towards ERK/MAPK pathways.

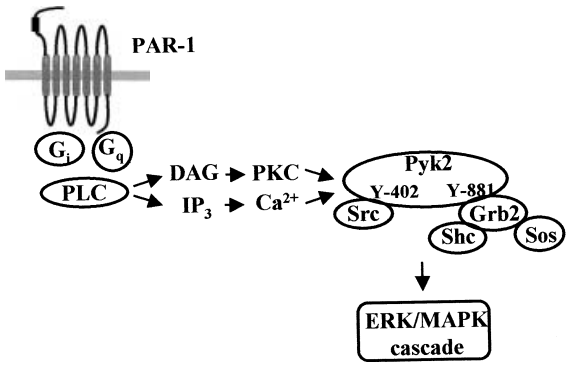

Fig. 7 Scheme showing involvement of proline-rich tyrosine kinase 2 (Pyk2) in signal transduction operated by protease-activated receptor (PAR)-1 in rat astrocytes. PAR-1 activation leads to Ca^{2+} mobilization and protein kinase C (PKC) activation, resulting in phosphorylation of Pyk2 and recruitment of Src tyrosine kinase and adapter protein growth factor receptor-bound protein (Grb)2, leading ultimately to extracellular signal-regulated kinase (ERK)1/2-mediated proliferation. The latter has been evaluated in detail recently (Wang *et al.* 2002b). DAG, Diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; Shc, adapter protein in the Ras (small GTPase) pathway; Sos, son of sevenless, Ras guanine nucleotide-releasing factor.

Besides being a regulator for ERK1/2 activation, Pyk2 may also be connected to other downstream transducers, such as c-Jun N-terminal kinase (JNK), by recruiting other adapter proteins, such as $p130^{Cas}$ and Crk (Blaukat *et al*. 1999). However, this possibility seems to be cell type-specific and agonist-dependent, since work from our laboratory has shown that PAR-1 activation in rat astrocytes fails to induce JNK phosphorylation (Wang and Reiser, unpublished data). Therefore, it is conceivable that thrombin-induced Pyk2 phosphorylation is not coupled to JNK activation in rat astrocytes.

In summary, we have demonstrated the expression and activation of Pyk2 in rat astrocytes. We suggest that Pyk2 may represent the tyrosine kinase which links the PAR-1-dependent increase in cytosolic Ca^{2+} and PKC activation to the Src tyrosine kinase-mediated ERK/MAPK pathway. Thrombin has been implicated in cellular events as diverse as neuroprotection and neurodegeneration in the brain (Striggow *et al*. 2000). Thus, understanding the role of Pyk2 in thrombin signal transduction may provide insights into the mechanisms that regulate the cellular consequences elicited by thrombin in the CNS.

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References

Avraham S., London R., Fu Y., Ota S., Hiregowdara D., Li J., Jiang S., Pasztor L. M., White R. A. and Groopman J. E. (1995) Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *J. Biol. Chem.* **270**, 27 742–27 751.

Bajetto A., Barbero S., Bonavia R., Piccioli P., Pirani P., Florio T. and Schettini G. (2001) Stromal cell-derived factor-1alpha induces astrocyte proliferation through the activation of extracellular signal-regulated kinases 1/2 pathway. *J. Neurochem.* **77**, 1226–1236.

Berridge M. J., Bootman M. D. and Lipp P. (1998) Calcium — a life and death signal. *Nature* **395**, 645–648.

Blaukat A., Ivankovic-Dikic I., Gronroos E., Dolfi F., Tokiwa G., Vuori K. and Dikic I. (1999) Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. *J. Biol. Chem.* **274**, 14 893–14 901.

Carpenter G. (1999) Employment of the epidermal growth factor receptor in growth factor-independent signaling pathways. *J. Cell. Biol.* **146**, 697–702.

Cazaubon S., Chaverot N., Romero I. A., Girault J. A., Adamson P., Strosberg A. D. and Couraud P. O. (1997) Growth factor activity of endothelin-1 in primary astrocytes mediated by adhesion-dependent and -independent pathways. *J. Neurosci.* **17**, 6203–6212.

Chen Y., Grall D., Salcini A. E., Pelicci P. G., Pouyssegur J. and Van Obberghen-Schilling E. (1996) Shc adaptor proteins are key transducers of mitogenic signaling mediated by the G protein-coupled thrombin receptor. *EMBO J.* **15**, 1037–1044.

Chiu T., Wu S. S., Santiskulvong C., Tangkijvanich P., Yee H. F. Jr and Rozengurt E. (2002) Vasopressin-mediated mitogenic signaling in intestinal epithelial cells. *Am. J. Physiol. Cell Physiol.* **282**, C434–C450.

Coughlin S. R. (1994) Protease-activated receptors start a family. *Proc. Natl Acad. Sci. USA* **91**, 9200–9202.

Daub H., Weiss F. U., Wallasch C. and Ullrich A. (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379**, 557–560.

Daub H., Wallasch C., Lankenau A., Herrlich A. and Ullrich A. (1997) Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.* **16**, 7032–7044.

Della Rocca G. J., van Biesen T., Daaka Y., Luttrell D. K., Luttrell L. M. and Lefkowitz R. J. (1997) Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of G_i- and G_q-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. *J. Biol. Chem.* **272**, 19 125–19 132.

Dikic I., Tokiwa G., Lev S., Courtneidge S. A. and Schlessinger J. (1996) A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383**, 547–550.

Earp H. S., Huckle W. R., Dawson T. L., Li X., Graves L. M. and Dy R. (1995) Angiotensin II activates at least two tyrosine kinases in rat liver epithelial cells. Separation of the major calcium-regulated tyrosine kinase from p125FAK. *J. Biol. Chem.* **270**, 28 440–28 447.

Ehrenreich H., Costa T., Clouse K. A., Pluta R. M., Ogino Y., Coligan J. E. and Burd P. R. (1993) Thrombin is a regulator of astrocytic endothelin-1. *Brain Res.* **600**, 201–207.

Felsch J. S., Cachero T. G. and Peralta E. G. (1998) Activation of protein tyrosine kinase PYK2 by the m1 muscarinic acetylcholine receptor. *Proc. Natl Acad. Sci. USA* **95**, 5051–5056.

Feng D. M., Veber D. F., Connolly T. M., Condra C., Tang M. J. and Nutt R. F. (1995) Development of a potent thrombin receptor ligand. *J. Med. Chem.* **38**, 4125–4130.

Grabham P. and Cunningham D. D. (1995) Thrombin receptor activation stimulates astrocyte proliferation and reversal of stellation by distinct pathways: involvement of tyrosine phosphorylation. *J. Neurochem.* **64**, 583–591.

Griffin W. S., Stanley L. C., Ling C., White L., MacLeod V., Perrot L. J., White C. L. and Araoz C. (1989) Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *Proc. Natl Acad. Sci. USA* **86**, 7611–7615.

Gschwind A., Zwick E., Prenzel N., Leserer M. and Ullrich A. (2001) Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene* **20**, 1594–1600.

Hollenberg M. D. (2002) PARs in the stars: proteinase-activated receptors and astrocyte function. Focus on 'Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways'. *Am. J. Physiol. Cell Physiol.* **283**, C1347–C1350.

Huckle W. R., Dy R. C. and Earp H. S. (1992) Calcium-dependent increase in tyrosine kinase activity stimulated by angiotensin II. *Proc. Natl Acad. Sci. USA* **89**, 8837–8841.

Kawabata A., Saifeddine M., Al Ani B., Leblond L. and Hollenberg M. D. (1999) Evaluation of proteinase-activated receptor-1 (PAR1) agonists and antagonists using a cultured cell receptor desensitization assay: activation of PAR2 by PAR1-targeted ligands. *J. Pharmacol. Exp. Ther.* **288**, 358–370.

Keogh R. J., Houliston R. A. and Wheeler Jones C. P. (2002) Thrombin-stimulated Pyk2 phosphorylation in human endothelium is dependent on intracellular calcium and independent of protein kinase C and Src kinases. *Biochem. Biophys. Res. Commun.* **294**, 1001–1008.

Lev S., Moreno H., Martinez R., Canoll P., Peles E., Musacchio J. M., Plowman G. D., Rudy B. and Schlessinger J. (1995) Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature* **376**, 737–745.

Litvak V., Tian D., Shaul Y. D. and Lev S. (2000) Targeting of PYK2 to focal adhesions as a cellular mechanism for convergence between integrins and G protein-coupled receptor signaling cascades. *J. Biol. Chem.* **275**, 32 736–32 746.

Martinez-Salgado C., Rodriguez-Barbero A., Tavares P., Eleno N. and Lopez-Novoa J. M. (2000) Role of calcium in gentamicin-induced mesangial cell activation. *Cell. Physiol. Biochem.* **10**, 65–72.

Murasawa S., Mori Y., Nozawa Y. *et al.* (1998) Role of calcium-sensitive tyrosine kinase Pyk2/CAKbeta/RAFTK in angiotensin II induced Ras/ERK signaling. *Hypertension* **32**, 668–675.

Neveu I., Jehan F., Jandrot Perrus M., Wion D. and Brachet P. (1993) Enhancement of the synthesis and secretion of nerve growth factor in primary cultures of glial cells by proteases: a possible involvement of thrombin. *J. Neurochem.* **60**, 858–867.

Pierce K. L., Luttrell L. M. and Lefkowitz R. J. (2001) New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene* **20**, 1532–1539.

Prenzel N., Zwick E., Daub H., Leserer M., Abraham R., Wallasch C. and Ullrich A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884–888.

Rocic P., Govindarajan G., Sabri A. and Lucchesi P. A. (2001) A role for PYK2 in regulation of ERK1/2 MAP kinases and PI 3-kinase by ANG II in vascular smooth muscle. *Am. J. Physiol. Cell Physiol.* **280**, C90–C99.

Sabri A., Govindarajan G., Griffin T. M., Byron K. L., Samarel A. M. and Lucchesi P. A. (1998) Calcium- and protein kinase C-dependent

activation of the tyrosine kinase PYK2 by angiotensin II in vascular smooth muscle. *Circ. Res.* **83**, 841–851.

Sasaki H., Nagura K., Ishino M., Tobioka H., Kotani K. and Sasaki T. (1995) Cloning and characterization of cell adhesion kinase beta, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. *J. Biol. Chem.* **270**, 21 206–21 219.

Schaller M. D. and Sasaki T. (1997) Differential signaling by the focal adhesion kinase and cell adhesion kinase beta. *J. Biol. Chem.* **272**, 25 319–25 325.

Striggow F., Riek M., Breder J., Henrich Noack P., Reymann K. G. and Reiser G. (2000) The protease thrombin is an endogenous mediator of hippocampal neuroprotection against ischemia at low concentrations but causes degeneration at high concentrations. *Proc. Natl Acad. Sci. USA* **97**, 2264–2269.

Tokiwa G., Dikic I., Lev S. and Schlessinger J. (1996) Activation of Pyk2 by stress signals and coupling with JNK signaling pathway. *Science* **273**, 792–794.

Ubl J. J. and Reiser G. (1997) Characteristics of thrombin-induced calcium signals in rat astrocytes. *Glia* **21**, 361–369.

Ubl J. J., Sergeeva M. and Reiser G. (2000) Desensitisation of protease-activated receptor-1 (PAR-1) in rat astrocytes: evidence for a novel mechanism for terminating Ca^{2+} signalling evoked by the tethered ligand. *J. Physiol.* **525**, 319–330.

Vaughan P. J., Su J., Cotman C. W. and Cunningham D. D. (1994) Protease nexin-1, a potent thrombin inhibitor, is reduced around cerebral blood vessels in Alzheimer's disease. *Brain. Res.* **668**, 160–170.

Wang H., Ubl J. J. and Reiser G. (2002a) The four subtypes of protease-activated receptors, co-expressed in rat astrocytes, evoke different physiological signaling. *Glia* **37**, 53–63.

Wang H., Ubl J. J., Stricker R. and Reiser G. (2002b) Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways. *Am. J. Physiol. Cell Physiol.* **283**, C1351–C1364.

Wilm M., Shevchenko A., Houthaeve T., Breit S., Schweigerer L., Fotsis T. and Mann M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**, 466–469.

Wu S. S., Chiu T. and Rozengurt E. (2002) ANG II and LPA induce Pyk2 tyrosine phosphorylation in intestinal epithelial cells: role of Ca^{2+}, PKC, and Rho kinase. *Am. J. Physiol. Cell Physiol.* **282**, C1432–C1444.

Yamaguchi M., Tanaka T., Waki M., Kitanaka A., Kamano H., Kubota Y., Ohnishi H., Takahara J. and Irino S. (1997) Antisense src expression inhibits tyrosine phosphorylation of Shc and its association with Grb2 and Sos which leads to MAP kinase activation in U937 human leukemia cells. *Leukemia* **11**, 497–503.