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Pharmacological characterization of protease-activated receptor (PAR-1) in rat astrocytes

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Abstract

The proteolytic action of thrombin on its receptor (protease-activated receptor-1 or PAR-1) results in a conformational change in which the new N-terminal sequence auto-activates the receptor. Peptide analogs of this N-terminal sequence (TRAPs) are able to mimic the effect of thrombin and an extensive search has led to the definition of the structural requirement for the agonist and antagonist activity on thrombin receptors in several peripheral systems. Thrombin plays an important role in central and peripheral nervous system development and PAR-1 is present in neurons and astrocytes. We have now characterized thrombin receptors pharmacologically in cultured rat astrocytes by using [^{3}H]thymidine incorporation and reversal of stellation induced by Bt_2cAMP as end-points. Thrombin increased [^{3}H]thymidine incorporation into DNA with an EC_{50} of 1 nM and induced a complete reversion of cell stellation. The effects of thrombin on [^{3}H]thymidine incorporation were mimicked by TRAP-14 (EC_{50} = 3 μM) and a peptide containing non-natural amino acids Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr-NH_2 (A6Y; EC_{50} = 0.8 μM). Similarly, these two peptides reversed Bt_2cAMP-induced stellation. The effect of thrombin, TRAP-14 and A6Y on [^{3}H]thymidine incorporation into DNA was significantly prevented by L9R, a 9-amino-acid peptide (Leu-Val-Arg-D-Cys-Gly-Lys-His-Ser-Arg; IC_{50} = 180 μM against thrombin and TRAP-14 and 800 μM against A6Y) previously described as an antagonist in human platelet aggregation. L9R antagonized also thrombin effects on astrocyte morphology. These results demonstrate that rat astrocytes express PAR-1 receptors which are pharmacologically similar to those previously characterized in human platelets.

Keywords: Thrombin; Protease-activated receptor; Astrocyte culture

1. Introduction

Thrombin is a serine protease involved in the blood coagulation cascade where it converts soluble fibrinogen to insoluble fibrin. Thrombin plays also a role in platelet and endothelial cell activation, smooth muscle cell and astrocyte proliferation and bone resorption. Thrombin exerts most of its cellular effects through the proteolytic activation of a cell surface receptor (protease-activated receptor-1 or PAR-1) (Rasmussen et al., 1991; Vu et al., 1991; Zhong et al., 1992). This receptor, discovered by direct expression cloning of mRNA, has been detected on human embryonic lung cells (HEL), hamster fibroblasts, and rat aortic smooth muscle cells, and is a member of the 7 transmembrane-spanning domain receptor family coupled to a G-protein (Rasmussen et al., 1991; Vu et al., 1991).

Thrombin activates its receptor by first associating with the hirudin-like extracellular domain of the receptor (receptor amino acids 52–69) and then cleaving off a N-terminal 41-amino-acid peptide, exposing a new N-terminus on the receptor. The newly exposed N-terminus (tethered ligand) then interacts with another, distant part of the receptor, resulting in activation (Vu et al., 1991). Remarkably, peptide analogs to the new amino-terminal sequence of the receptor (TRAPs), beginning with serine and ranging from 5 to 14 amino acids, have been found to mimic the action of thrombin in a variety of target tissues ranging from platelets to neuronal cells, albeit at concentrations 1000-fold higher than thrombin (Vu et al., 1991; Vouret-Craviari et al., 1992; Ogletree et al., 1993; Debeir et al., 1996a,b). The involvement of PAR-1 in neuronal differentiation is supported by multiple lines of evidence. PAR-1 and prothrombin mRNA are expressed by neurons and astrocytes in several rat brain regions (Dihanich et al., 1991; Niclou et al., 1994; Soifer et al., 1994; Weinstein et al., 1995). Moreover, thrombin and TRAP-14 have trophic

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effects on cholinergic neurons in culture which are dependent on the presence of astrocytes (Debeir et al., 1996a).

It is now well established that thrombin and TRAP induce proliferation and reverse stellation of cultured astrocytes (Cavanaugh et al., 1990; Nelson and Siman, 1990). One-hundred-fold higher concentrations of thrombin are required for cell proliferation compared to stellation reversal. Because low and high concentrations of thrombin induced differential response of astrocytes, it has been suggested that thrombin effects on astrocytes occur through different pathways (Grabham and Cunningham, 1995; Debeir et al., 1996b). It has been reported that the action of thrombin on astrocyte proliferation and stellation was prevented by thrombin inhibitors such as protease nexin-1 (Cavanaugh et al., 1990; Beecher et al., 1994). However, this serpin directly inhibits the enzyme and hence, does not provide information about the involvement of PAR-1 in the effects of thrombin on astrocytes. A second member of the protease-activated receptor family has been recently identified (Nystedt et al., 1994). This receptor (PAR-2) is preferentially activated by trypsin which creates a new N-terminal peptide (tethered ligand) different from that of PAR-1.

The identification of the human thrombin receptor has prompted considerable interest in the development of PAR-1 agonists and antagonists (Seiler, 1996) that can mimic or prevent the cellular effects of thrombin without affecting fibrin formation. Chemically modified peptides with a much greater potency than TRAP-14 have been described. This is the case for Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr-NH_2 (A6Y), that activates human platelets with an EC_{50} of 0.01 μM, 1000-fold lower than TRAP-14 (Feng et al., 1995). On the other hand, several peptides containing non-natural amino acids have demonstrated antagonistic activity. This is the case for 2-phenoxybutyryl-Cha-Cha-(*N*-Me-Arg)-Arg-NH_2 (phenoxy peptide) and 3-mercapto-propionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH_2 (Mpa peptide) that prevent thrombin-induced activation of human platelets (Scarborough, 1994; Seiler et al., 1995). Mpa peptide is also active at inhibiting thrombin stimulation of phospholipase A_2 and Na^+/H^+ exchange (Seiler et al., 1995). Another peptide which contains natural amino acids has also been demonstrated to be a PAR-1 antagonist. This peptide, Leu-Val-Arg-D-Cys-Gly-Lys-His-Ser-Arg (L9R), not only inhibits thrombin-induced platelet aggregation (Maraganore and Frelinger, 1993) but also prevents the effect of this protease on smooth muscle and CCL-39 cell proliferation (D. O'Brien, personal communication) and septal cholinergic neuron differentiation (Debeir et al., 1996a).

The main objective of this work was to pharmacologically characterize PAR-1 receptors in cultured rat astrocytes. To this end, we have compared the effects of the above-mentioned agonists and antagonists on two cellular responses: reversal of stellation and proliferation. Moreover, in order to ascertain if astrocytes express functional PAR-2 receptors, we have also determined the effect of the N-terminal sequence of the activated PAR-2 Ser-Leu-Ile-Gly-Arg-Leu (SLIGRL) on these cellular responses.

2. Materials and methods

2.1. Materials

Timed pregnant Sprague-Dawley rats were obtained from Janvier (CERJ, Le Genest, France); Blutex nylon screens were from Tissage Tissu Technique. Phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), Ham's nutrient mixture F12, HBSS, penicillin and streptomycin, and fetal bovine serum were from Gibco. Tissue culture dishes and immunoplates were from Costar and Nunc, respectively.

[^{3}H]Thymidine (6.7 Ci/mmol) was purchased from New England Nuclear. Human purified α-thrombin was from Euromedex. The PAR-1 agonist peptide TRAP-14 (Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe) and the PAR-2 agonist peptide (Ser-Leu-Ile-Gly-Arg-Leu) were from Bachem. Thrombin receptor antagonist peptides, Leu-Val-Arg-D-Cys-Gly-Lys-His-Ser-Arg (L9R), 2-phenoxybutyryl-Cha-Cha-(*N*-Me-Arg)-Arg and 3-mercapto-propionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys (Mpa peptide) and the thrombin receptor agonist Ala-Phe(*p*-F)-Arg-Cha-HArg-Tyr-NH_2 (A6Y) were prepared by solid-phase peptide synthesis.

2.2. Astrocyte cultures

Primary cultures of astrocytes were prepared as previously described by Vigé et al. (1991). Briefly, cerebral cortices from 1-day-old rat pups were rapidly dissected out into sterile Leibovitz's L-15 medium. Cells were mechanically dissociated through 83 μm sterile Blutex nylon screens into 10 ml of culture medium. The medium consisted of DMEM/F12 (1:1), 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. After trituration to obtain a homogeneous preparation, medium was added to give a suspension (one cortex for 30 ml of medium), and cells were plated onto 12-well Corning dishes (10^6 cells/well/ml). Cells were grown for 7–8 days at 37°C in a water-saturated air environment containing 5% CO_2. Culture medium was changed every 3 days of culture. At 7–8 day in vitro, the cultures consisted of a layer of cells (80–90% confluent) of which 90–95% were astrocytes, as shown by glial fibrillary acidic protein immunocytochemical staining (Čarman-Kržan et al., 1991). A minor population of round phase-bright cells which are probably oligodendrocytes grew on top of the monolayer.

After 7–8 days in vitro, astroglial cells were treated with indicated compounds. To avoid possible artefacts due to the presence of thrombin in serum, serum-free medium was used.

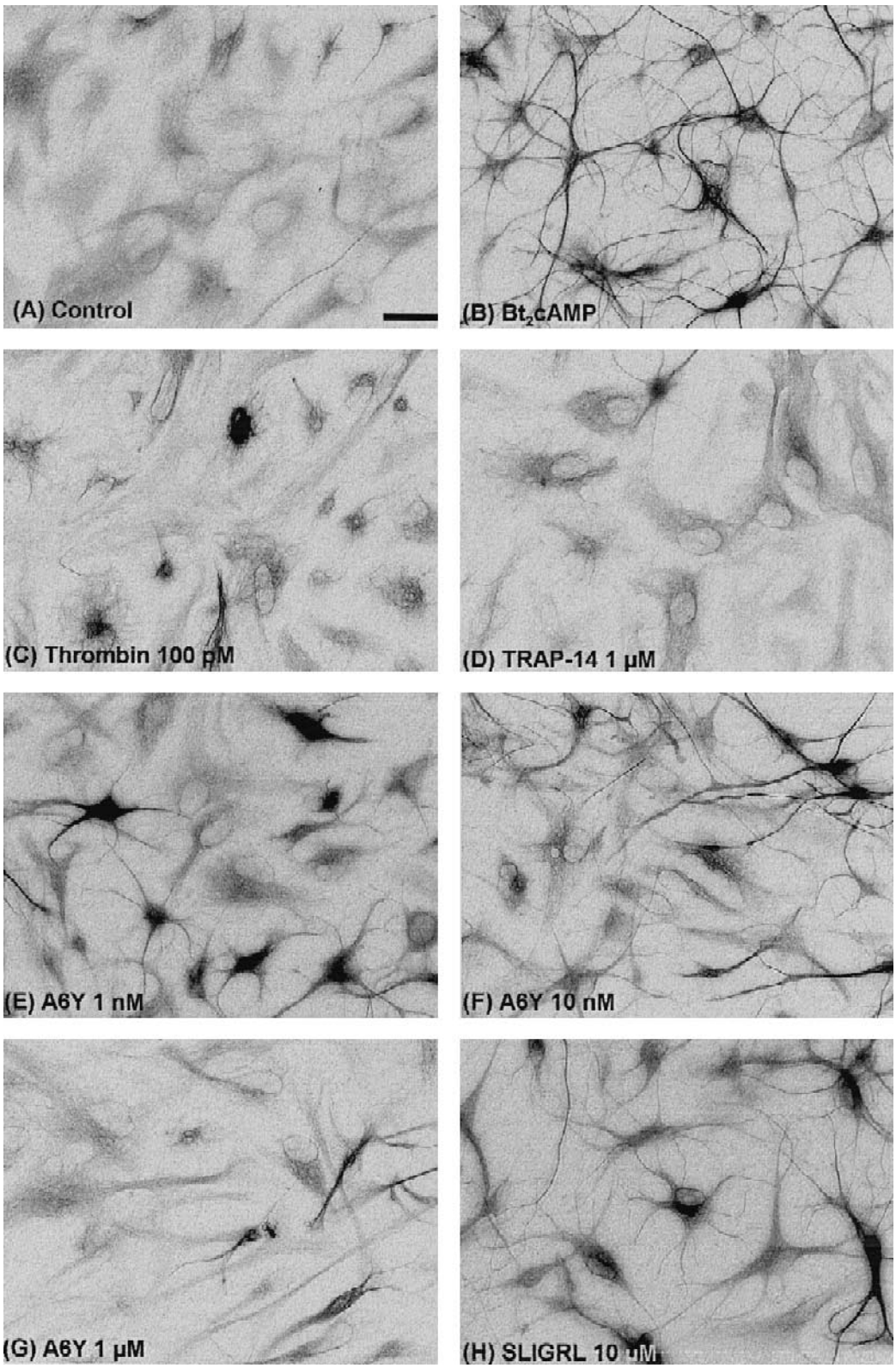

Fig. 1. Thrombin and peptide agonist effects on astrocyte morphology. (A) Astrocytes maintained for 4 h in serum-free media in the absence of Bt_2cAMP. (B) Cells treated with 500 µM Bt_2cAMP for 4 h. (C, D, E, F, G and H) Astrocytes treated with indicated compounds for 1 h following a 4 h period of 500 µM Bt_2cAMP treatment. After incubation, cells were fixed and immunolabeled for GFAP as described in Section 2. Negative prints; bar = 25 µm.

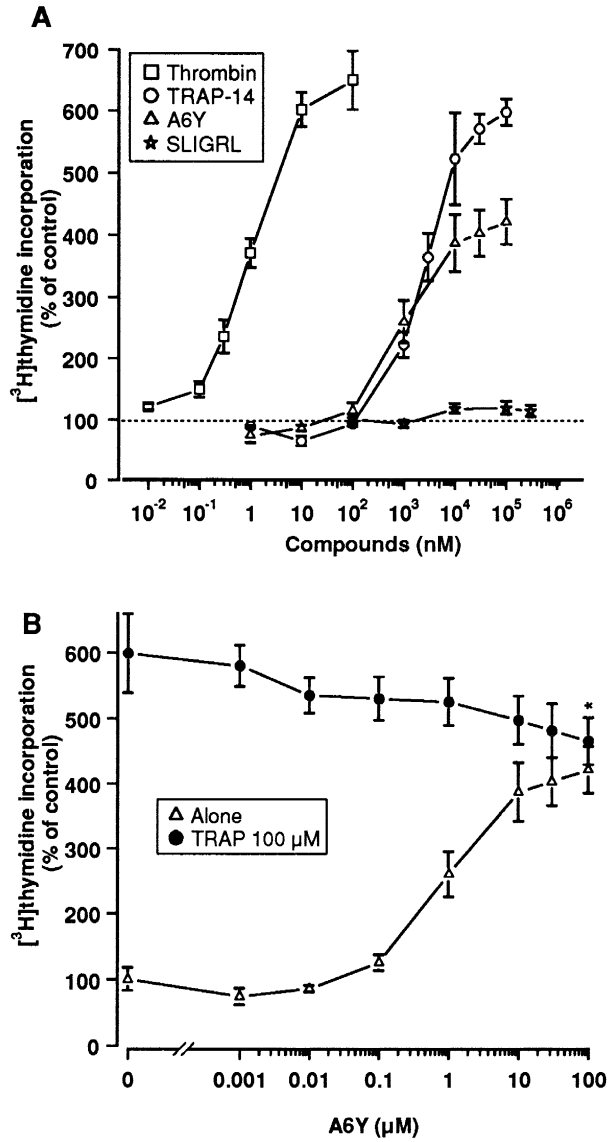

Fig. 2. Effect of PAR-1 and PAR-2 agonists on [^{3}H]thymidine incorporation by cultured astrocytes. (A) Astrocytes were cultured in the presence of the indicated concentrations of thrombin, TRAP-14, A6Y or SLIGRL for 48 h. (B) Astrocytes were treated for 48 h with A6Y at the indicated concentrations and 100 μM TRAP-14. Cells were pulsed with 1 μCi/ml of [^{3}H]thymidine for the final 16 h of culture. Values are means with S.E.M. of four separate experiments. $^*P < 0.05$ vs. TRAP-14 alone.

2.3. Measurement of DNA synthesis

DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material. Astrocytes were stimulated for 48 h by different agents in a serum-free medium consisting of DMEM/F12 (1:1), 100 units/ml penicillin and 100 μg/ml streptomycin. [^{3}H]Thymidine (1 μCi/ml) was added in the last 16 h. After removing the medium and washing with HBSS, cells were fixed with trichloroacetic acid (15%), [^{3}H]thymidine incorporated into trichloroacetic acid-precipitable material was recovered with 1 M NaOH and measured in a scintillation counter.

2.4. Reversion of stellation

After 7 days in vitro, cell stellate morphology was induced by treatment with 500 μM of Bt_2cAMP in serum-free medium for a period of 4 h. The different test substances were then added to the culture medium. After treatment, cells were fixed with 100% methanol (−20°C, 15 min), washed three times with phosphate-buffered saline (PBS) and incubated with a monoclonal anti-glial fibrillary protein (anti-GFAP) antibody conjugated to Cy3 (Sigma Chemical) at 1/200 dilution in antibody diluent (Dako) for 1 h. After washing, coverslips were mounted using fluorescent mounting medium (Dako) and viewed on an Olympus microscope. Cells were scored for the presence of GFAP staining and morphology.

2.5. Statistical analyses

Statistical analyses were performed using Duncan's multiple range test.

3. Results

3.1. Differential effects of peptide agonists of PAR-1 and PAR-2 on astrocytes

In serum-containing medium, cortical astrocytes display a flattened morphology (Fig. 1A). Removal of serum and

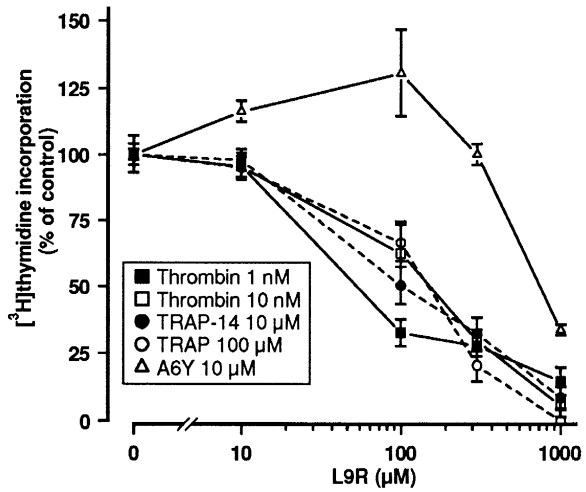

Fig. 3. Inhibition of PAR-1 agonist-induced DNA synthesis in astrocytes by L9R. Cells were treated with L9R (added 5 min before the agonists) at the indicated concentrations and either with thrombin, TRAP-14 or A6Y for 48 h. Cells were pulsed with 1 μCi/ml of [^{3}H]thymidine for the final 16 h of culture. Values are means with S.E.M. of four separate experiments.

addition of Bt_2cAMP (500 μM) for 4 h, causes astrocyte stellation. Most astrocytes display a star-like appearance, with rounded cell bodies and long thin processes (Fig. 1B). Addition of thrombin (100 pM) or TRAP-14 (1 μM) results in a complete reversion to the flattened shape (Fig. 1C, D).

Similar effects were observed after addition of the novel peptide agonist A6Y that induced a concentration-dependent astrocyte flattening (Fig. 1E, F, G). Reversal of stellation is complete after 1 h at 1 μM A6Y (Fig. 1G) or after 5 h at 1 nM A6Y (data not shown). In contrast, the PAR-2-tethered agonist SLIGRL (10 μM) did not affect the morphology of the astrocytes (Fig. 1H). SLIGRL from 100 nM to 100 μM was also inactive when incubated for a longer time (5 h; data not shown).

As previously reported (Debeir et al., 1996b), thrombin and TRAP-14 induce a dose-dependent increase in [^{3}H]thymidine incorporation (EC_{50} = 1 nM and +550% at 100 nM for thrombin; EC_{50} = 3 μM and +500% at 100 μM for TRAP-14; Fig. 2A), reflecting astrocyte proliferation. The peptide agonist A6Y also induced a concentration-dependent increase in DNA synthesis (EC_{50} = 800 nM and +300% at 100 μM; Fig. 2A). A6Y partially reversed the maximal effect of TRAP-14 (Fig. 2B). In contrast, the PAR-2-tethered ligand SLIGRL had no effect on DNA synthesis (Fig. 2A).

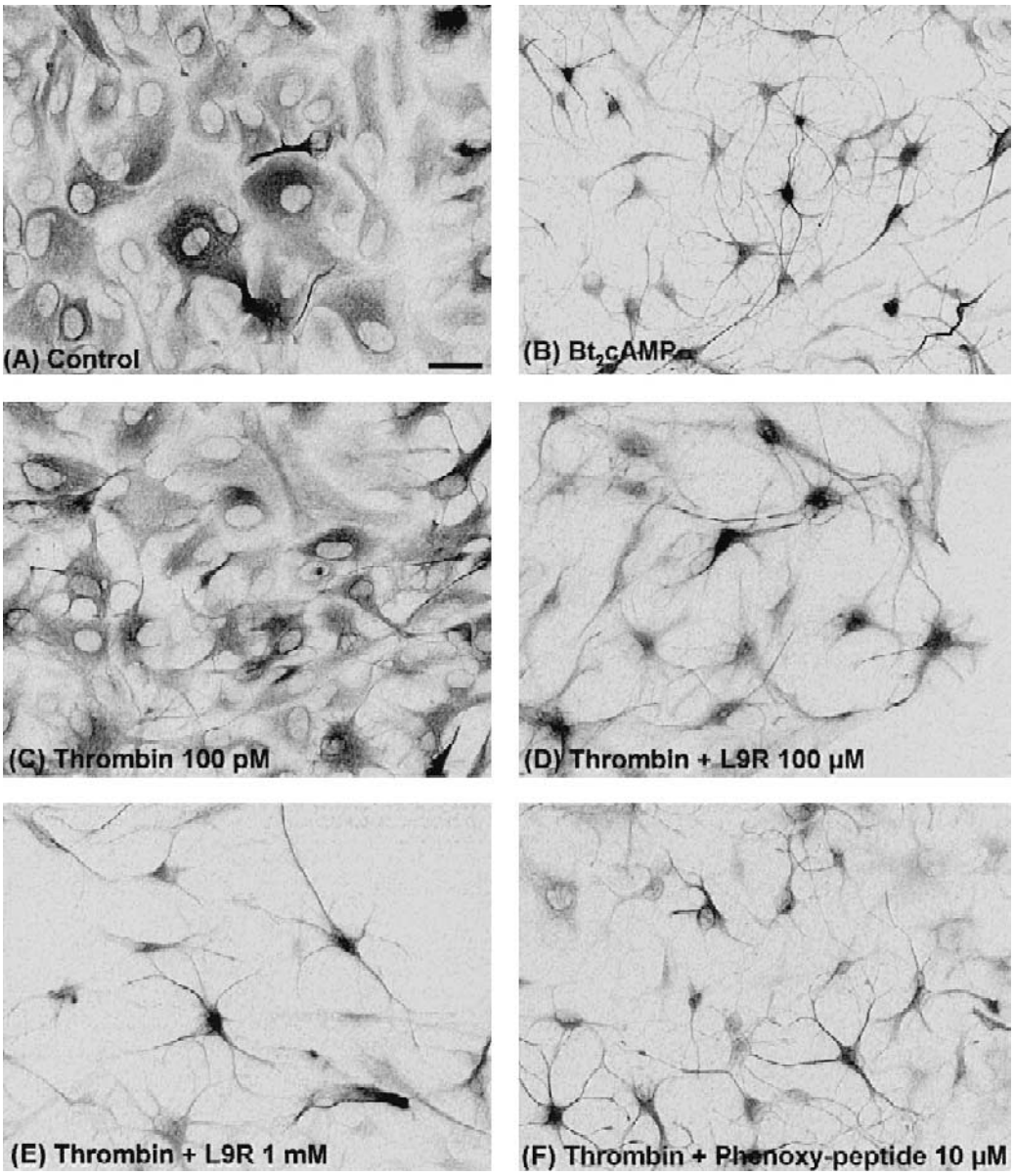

Fig. 4. Inhibitory effects of PAR-1 antagonists on thrombin-induced reversal of astrocyte stellation. (A) Astrocytes maintained for 4 h in serum-free media in the absence of Bt_2cAMP. (B) Cells treated with 500 μM Bt_2cAMP for 4 h. (C, D, E and F) Astrocytes treated with indicated compounds for 1 h following a 4 h period of 500 μM Bt_2cAMP treatment. After incubation, cells were fixed and immunolabeled for GFAP as described in Section 2. Negative prints; bar = 25 μm.

3.2. Inhibition of thrombin effects on astrocytes by PAR-1 antagonists

L9R, an antagonist of platelet PAR-1, has no effect per se on basal proliferation (data not shown). However, this peptide inhibits in a concentration-dependent manner thrombin, TRAP-14- and A6Y-induced [^{3}H]thymidine incorporation (Fig. 3). The IC_{50} values of L9R at inhibiting thrombin (10 nM), TRAP-14 (100 μM) and A6Y (10 μM) were 185 μM, 170 μM and 800 μM, respectively. In contrast, 2-phenoxybutyryl-Cha-Cha-(*N*-Me-Arg)-Arg (phenoxy peptide) (0.1–100 μM) and 3-mercapto-propionyl-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys (Mpa peptide; 0.1–30 μM), two other antagonists at the platelet level, had no effect on thrombin-induced proliferation. Higher concentrations of phenoxy peptide and Mpa peptide could not be used because they were toxic for astrocytes after several hours of treatment (microscopic observations). In contrast, L9R, up to 1 mM, had no toxic effect.

The activity of these compounds at preventing thrombin-induced reversion of stellation has also been studied. L9R (100 μM) and the phenoxy peptide (10 μM) have no effect in basal conditions but reverse the effects of 100 pM thrombin (Fig. 4). The effect of the Mpa peptide could not be tested, because toxic effects appeared shortly after its addition to the incubation medium.

4. Discussion

In the present work we have pharmacologically characterized thrombin receptors (PAR-1) in astrocytes, using several peptides recently described as agonists or antagonists of human platelet PAR-1.

Our results clearly demonstrate that the effects of thrombin on astrocyte proliferation and morphology are mediated by PAR-1, since they are mimicked by two peptides agonists of this receptor (TRAP-14 and A6Y) and blocked by L9R, previously described as an antagonist in human platelet (Maraganore and Frelinger, 1993). In contrast, PAR-2 does not seem to be involved in these responses since SLIGRL, its tethered agonist, fails to mimic these astrocyte responses. Consistent with our results, Nelson and Siman (1990) and Cavanaugh et al. (1990) have reported that in astrocytes trypsin was ineffective in reversing Bt_2cAMP-induced morphological differentiation. Although it has been demonstrated that trypsin is able to stimulate astrocyte proliferation (Perraud et al., 1987), our observations suggest that this effect is not mediated by PAR-2.

It is interesting to compare the effects of PAR-1 peptide agonists and antagonists on platelets and astrocytes (Table 1). In contrast to human platelets, where it appears that the potency of A6Y at inducing aggregation was 1000-fold higher than that of TRAP-14 (Feng et al., 1995), the potencies of A6Y and TRAP-14 on rat astrocyte proliferation are similar. At the concentration of 1 μM, both peptide agonists of PAR-1 reversed completely Bt_2cAMP-induced stellation of astrocytes, whereas at higher concentrations (10 μM), A6Y has a lower efficacy than TRAP-14 on cell proliferation.

These differences in potency and sensitivity may relate to the fact that different transduction mechanisms requiring different degrees of PAR-1 activation are involved in these biological effects (Debeir et al., 1996b; Grabham and Cunningham, 1995), or alternatively to the different structural requirement for the activation of rat and human PAR-1 (for review Grand et al., 1996).

Among the three peptide antagonists active on platelets (Table 1) only one of them prevented thrombin-induced proliferation and two antagonized stellation reversal. These differences may relate to the experimental conditions. Indeed, measuring proliferation required a 48 h incubation

Table 1
Comparative effects of PAR-1 agonists and antagonists on astrocytes and platelets

Peptides	Previously defined as	Human platelet aggregation (EC_{50})	Rat astrocyte proliferation (EC_{50})	Rat astrocyte morphology [a]
TRAP-14	Agonist PAR-1	10 μM (Feng et al., 1995)	3 μM	Active
A6Y	Agonist PAR-1	0.01 μM (Feng et al., 1995)	0.8 μM	Active
SLIGRL	Agonist PAR-2	Inactive (Hwa et al., 1996)	Inactive	Inactive
		(IC_{50})	(IC_{50})	Inhibitory effect [b]
L9R	Antagonist PAR-1	30 μM (Maraganore and Frelinger, 1993)	180 μM	Active
Phenoxy peptide	Antagonist PAR-1	0.5 μM (Scarborough, 1994)	Inactive Toxic at 100 μM	Active
Mpa peptide	Antagonist PAR-1	80 μM (Seiler et al., 1995)	Inactive Toxic at 30 μM	Toxic

[a] Effect of peptides on Bt_2cAMP-induced stellation. [b] Inhibitory effect of peptide on thrombin-induced reversal of astrocyte stellation.

and the experiments of morphology 1 h, whereas aggregation is a very rapid phenomenon. The longer incubation periods which result in cytotoxic effects may explain our inability to demonstrate the antagonistic activity of the phenoxy peptide on astrocyte proliferation, and the Mpa peptide on both events. Differences between rat and human PAR-1 may also provide a complementary or alternative explanation as discussed above for PAR-1 agonists.

Although activation of proliferation requires a thrombin or agonist concentration much higher than those required for stellation, both effects seem to be mediated by the same receptor, since they are prevented by the antagonist L9R. Induction of proliferation seems to require a much greater receptor activation and this may explain why A6Y behaves as a full agonist in the stellation paradigm but as a partial agonist at inducing proliferation.

L9R and A6Y are non-natural amino-acid-derived peptides that may be less susceptible to degradation than natural peptides. The availability of these compounds may help to further define the involvement of PAR-1 in central nervous system (CNS) development and regeneration, and to establish the potentialities of thrombin antagonists or partial agonists as novel therapeutics for neurodegenerative diseases.

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