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Reactive blue 2 inhibition of cyclic AMP-dependent differentiation of rat C6 glioma cells by purinergic receptor-independent inactivation of phosphatidylinositol 3-kinase

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Abstract

Cyclic AMP-dependent differentiation of rat C6 glioma cells into an astrocyte type II is characterized by inhibition of cell growth and induction of glial fibrillary acidic protein (GFAP) synthesis. Activation of the $P2Y_{12}$ receptor with 2-methylthioadenosine-5′-diphosphate inhibited β-adrenergic receptor-induced differentiation. The selective $P2Y_{12}$ receptor antagonist N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP abolished the receptor-mediated effect on differentiation. In contrast non-selective antagonists of P2Y receptors did not revert the inhibiting effect of the $P2Y_{12}$ receptor on differentiation. Reactive blue 2 (RB2), a potent $P2Y_{12}$ receptor antagonist, completely inhibited the synthesis of GFAP, while the P2Y receptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid were less efficient. However, although P2Y receptor antagonists inhibited GFAP synthesis to a different extent they were unable to relieve the growth inhibition that accompanied induction of differentiation, whereas stimulation of the $P2Y_{12}$ receptor with 2-methylthioadenosine-5′-diphosphate inhibited GFAP expression and restored cell proliferation. Assay of the activity of phosphatidylinositol 3-kinase (PI 3-K), an enzyme required for GFAP expression [J. Neurochem. 76 (2001) 610], showed that RB2 inhibited this enzyme after cellular uptake, while suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid inhibited PI 3-K to a lesser extent. The intracellular concentration of RB2 increased in time and attained the IC_{50} for PI 3-K inhibition (4 μM) after 40-min incubation with 50 μM RB2. In conclusion, cAMP-induced differentiation in C6 cells is inhibited by activation of the $P2Y_{12}$ receptor. In addition, synthesis of GFAP is also inhibited by cellular uptake of non-selective nucleotide receptor antagonists that inhibit PI 3-K, a kinase required for the cAMP-dependent induction of differentiation.

Keywords: $P2Y_{12}$ receptor; Purinergic receptor antagonists; Phosphatidylinositol 3-kinase; Glial fibrillary acidic protein; Differentiation; Rat C6 glioma

1. Introduction

Astrocytes, neuronal, and endothelial cells have been reported to release nucleotides by non-lytic mechanisms [1–3]. Accumulating evidence indicate that these nucleotides are stored in vesicles and that their secretion is regulated [4,5]. Released nucleotides and their hydrolysis products activate P2 and adenosine (P1) receptors that elicit a broad range of responses, including neurotransmission, calcium-mediated intercellular communication, protection in hypoxia and ischemia, trophic actions on the proliferation and differentiation of glial and neuronal

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Abbreviations: AR-C69931MX, N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP; dbcAMP, dibutyryl cAMP; CSLM, confocal laser scanning microscopy; GFAP, glial fibrillary acidic protein; MAPK, mitogen-activated protein kinase; MEM, minimal essential medium; 2MeSADP, 2-methylthioadenosine-5′-diphosphate; PI 3-K, phosphatidylinositol 3-kinase; PI(3)P, phosphatidylinositol 3-phosphate; PIPES, 1,4-piperazinediethanesulfonic acid; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; RB2, reactive blue 2.

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cells, etc. [5,6–9]. The varying effects of nucleotides on different cell types reflect the final outcome of expression of purine receptor subtypes and the modulating effect of cell surface-bound nucleotidases, e.g. ecto-ATPase (EC.3.6.1.3), ecto-apyrase (EC.3.6.1.5), ecto-nucleotide pyrophosphatase/phosphodiesterase (EC.3.6.1.9), and ecto-nucleotidase (EC.3.1.3.5) [10,11]. The activated P2 receptors are ligand-gated ion channels (P2X) or G protein-coupled receptors (P2Y) linked to activation of phospholipase C and/or activation or inhibition of adenylate cyclase [6]. These receptors are characterized by their pharmacological agonist profile and identified by the use of selective and non-selective receptor antagonists. To date some selective P2Y antagonists are already available, e.g. N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (MRS-2179) is specific for the $P2Y_1$ receptor, N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX) is specific for $P2Y_{12}$ and $P2Y_{13}$ receptors, but non-selective antagonists, like the anthraquinone RB2, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), suramin, etc. are still in use. The fact that these antagonists are also inhibitors of nucleotide metabolizing enzymes present at the cell surface and in the extracellular medium complicates the interpretation of the obtained data [12–14].

Rat C6 glioma cells have oligodendrocytic and astrocytic progenitor properties and are often used as biochemical model system for astrocytes. Differentiation towards an astrocyte type II is induced by elevation of the intracellular cAMP concentration upon stimulation of β-adrenergic receptors, positively coupled to adenylate cyclase, or by membrane-permeable cAMP analogues, such as dibutyryl cAMP (dbcAMP). The mechanism of differentiation does not require PKA activation, but is dependent on the activity of PI 3-K [15,16]. Upon induction of differentiation, C6 cells undergo morphological changes from a bipolar to a stellate shape concomitant with growth arrest and a shift in the expression of the intermediate filament protein vimentin towards GFAP [17,18].

C6 cells express the $P2Y_{12}$ receptor that is activated by 2-methylthioadenosine-5′-diphosphate (2MeSADP), a receptor agonist not hydrolyzed by the ecto-enzymes present on these cells [19,20]. Cyclic AMP increase, induced in these cells by β-adrenergic receptor stimulation with (−)-isoproterenol, is inhibited by simultaneous stimulation of the $P2Y_{12}$ receptor with 2MeSADP [21,22], suggesting that activation of the latter receptor may inhibit the cAMP-dependent induction of differentiation.

In this communication, we demonstrated that besides a $P2Y_{12}$ receptor-mediated inhibition of the cAMP-dependent induction of differentiation also non-selective P2Y receptor antagonists can suppress GFAP synthesis by a receptor-independent inhibition of PI 3-K, an enzyme required for induction of differentiation [16].

2. Methods

2.1. Materials

2MeSADP, dbcAMP, suramin, PPADS, RB2, and (−)-isoproterenol were from Sigma/RBI. The $P2Y_{12}$ receptor antagonist AR-C69931MX was a kind gift from AstraZeneca.

2.2. Cell culture

Rat C6 glioma cells (ATCC CCL 107) were obtained from ATCC and maintained in monolayer culture as described previously [23]. Experiments were performed on cells cultured in 96-well plates or 90 mm petri dishes and in serum-free chemically defined medium containing Ham's F10/minimal essential medium (MEM, 1:1, v/v), 2 mM L-glutamine, 1% (v/v), MEM vitamins (100×), 1% (v/v) MEM non-essential amino acids (100×), 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO), and 30 nM sodium selenite (Sigma/RBI). Cell numbers were measured in a hemocytometer after cell detachment with trypsin/EDTA in PBS (1:50, v/v).

2.3. Immunodetection of GFAP

C6 cells were cultivated in 96-well plates in serum-free chemically defined medium up to a density of approximately 10^5 cells/cm^2. Fifteen minutes before stimulation with 5 μM (−)-isoproterenol or 1 mM dbcAMP, P2Y receptor antagonists (50 μM) or PBS were added, and 5 min before stimulation with 5 μM (−)-isoproterenol or 1 mM dbcAMP, the P2Y receptor agonist 2MeSADP, UTP, or PBS was added, as indicated in the figure legends. After 48 hr, the medium was aspirated and 1 μL/10^3 cells SDS–PAGE sample buffer [6 mM Tris–HCl (pH 6.8), 0.5% (w/v) SDS, 10% (v/v) glycerol, 0.5% (v/v) 2-mercaptoethanol] was added. Samples were boiled for 5 min, and 20 μL were analyzed by SDS–PAGE on a 12.5% (w/v) polyacrylamide gel. Proteins were electroblotted overnight onto a nitrocellulose membrane (Hybond-C pure, Amersham Pharmacia Biotech). Immunodetection was performed using a polyclonal rabbit anti-cow GFAP antibody diluted 1:10,000 (Dakopatts). The nitrocellulose membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody diluted 1:40,000 and GFAP was visualized on X-OMAT blue film (KODAK) by enhanced chemiluminescence (Supersignal West Pico, Perbio Science). The visualized protein was quantified with the Geldoc 2000 system (Bio-Rad).

2.4. Immunofluorescence labeling

Cells were cultured on coverslips in chemically defined medium as described above up to a density of 8.10^4 cells/cm^2. Fifteen minutes before stimulation with 5 μM (−)-

isoproterenol or 1 mM dbcAMP, 50 μM P2Y receptor antagonist (RB2, suramin, PPADS) or PBS was added. After incubation for 48 hr, the medium was aspirated and the cells were fixed at room temperature with 0.5% (w/v) paraformaldehyde in cytoskeletal stabilizing buffer [127 mM NaCl, 50 mM KCl, 1.1 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM EGTA, 20 mM 1,4-piperazinediethanesulfonic acid (PIPES; pH 6.5), 5.5 mM glucose] for 15 min. After three rinses with buffer, aldehyde groups were reduced by two treatments with 50 mM NH_4Cl in cytoskeletal stabilizing buffer for 5 min. Cells were rinsed as before and permeabilized with buffer containing 0.3% (v/v) Triton X-100 for 5 min. After rinsing with buffer, the cells were incubated with buffer containing 3% (w/v) BSA for 1 hr at 37°. Coverslips were rinsed as before and incubated for 1 hr at 37° with 200 μL polyclonal rabbit anti-GFAP antibody (1:160; Dakopatts) diluted in buffer. Subsequently, coverslips were rinsed and incubated with 200 μL Cy3-labeled donkey anti-rabbit IgG antibody (1:100; Jackson Immunoresearch) in cytoskeletal stabilizing buffer. After the final washing steps, the coverslips were mounted in Vectashield (Vector Laboratories).

2.5. Confocal laser scanning microscopy (CSLM)

CLSM was done on a Bio-Rad MRC 600 (25 mW argon ion laser; Bio-Rad Laboratories) mounted on a Zeiss Axioskop equipped with a 63× (NA 1.25) oil immersion objective, using the BHS filter set (excitation 514 nm, beamsplitter 540 nm, emission LP 550 nm). To compare fluorescence intensities, identical microscope settings (laser intensity, pinhole, gain and black level) were used.

2.6. PI 3-K assay

Cells were washed with 5 mL ice-cold PBS, and collected by scraping and centrifugation at 400 *g* for 5 min. The pellet was resuspended in lysis buffer [20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 2 mM DTT, 1 mM PMSF, 1 mM Na-orthovanadate, 1% (v/v) NP-40, 10 mM iodoacetamide, 10 mM NaF, and a protease-inhibitor cocktail (Roche)] and shaken for 20 min at 4°. Subsequently, the lysate was centrifuged for 5 min at 12,000 *g* and the supernatant collected. After preclearence with protein G beads (Pierce), the supernatant was incubated for 2 hr with anti-PI 3-K p85 antibody (Upstate Biotechnology) and protein G beads were added. After 1 hr beads were collected, washed three times with 1 mL lysis buffer and two times with 50 mM Tris–HCl (pH 7.4), and assayed for PI 3-K activity.

Ten micrograms phosphatidylinositol and 20 μg phosphatidylserine, dissolved in $CH_3OH/CHCl_3$ (1:1, v/v), were evaporated under a flow of nitrogen in a hemolysis tube and suspended in 10 μL 50 mM Tris–HCl (pH 7.4). The mixture was vortexed for 1 min and sonicated before addition of 10 μCi [γ-^{32}P]ATP. The antibody–PI 3-K complex coupled to protein G beads was suspended in half the final volume of twice concentrated kinase buffer [100 mM Tris–HCl pH (7.4), 3 mM DTT, 200 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 200 μM ATP] and added to the lipid mixture. After incubation at 37° for 30 min the reaction was stopped with 1 mL CH_3OH/0.8 N HCl (1:1, v/v) and 0.5 mL $CHCl_3$ was added. The mixture was vortexed for 1 min and centrifuged at 200 *g*. The organic phase was collected and evaporated under a flow of nitrogen. Samples were redissolved in 70 μL $CH_3OH/CHCl_3$ (1:1, v/v) and spotted under nitrogen on an oxalate-precoated TLC plate. Precoating with oxalate was by overnight migration in oxalate buffer [H_2O/CH_3OH 3:2, 1.5% (w/v) K^+-oxalate, 2 mM EDTA] and subsequent heating at 100° for 15 min. After chromatography in $CHCl_3$/acetone/CH_3OH/acetic acid/H_2O (80:30:26:24:14, v/v/v/v/v), radioactive spots were visualized by phosphoimaging and quantified using the ImageQuant software (PhosphoImager SI, Amersham Pharmacia Biotech).

2.7. Immunodetection of PI 3-K

In each condition, one tenth of the immunoprecipitate was analyzed for total PI 3-K by Western blotting. The beads were boiled for 5 min in 20 μL SDS–PAGE sample buffer and proteins were analyzed by SDS–PAGE as described above. Immunodetection was performed using the anti-PI 3-K p85 antibody (Upstate Biotechnology) diluted 1:3000. The nitrocellulose membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody diluted 1:40,000 and the p85 subunit visualized on X-OMAT blue film (KODAK) by enhanced chemiluminescence (Supersignal West Pico, Perbio Science). The visualized protein was quantified with the Geldoc 2000 system (Bio-Rad).

2.8. Analysis of radioactive spots

The phosphatidylinositol 3-phosphate [PI(3)P] spots were analyzed with a modified method of Auger *et al.* [24]. Briefly, radioactive spots were scraped into a hemolysis tube containing 800 μL methylamine buffer [methylamine 10.7%, CH_3OH 45.8%, *n*-butanol 11.4% (v/v)]. Silica was suspended by repeated pipetting. After incubation for 1 hr at 53°, silica was dried under nitrogen and the deacylated lipids dissolved in 1.4 mL water. Silica was removed by filtration on a 0.45 μm filter before analysis of glyceroinositol-phosphates by anion-exchange HPLC on a Partisphere Sax column (Whatman Int. Ltd). A gradient from 0 to 2.0 M $(NH_4)_2HPO_4$ (pH 3.35) was developed in 115 min. Dual pumps were used to establish the gradient (pump A, H_2O; pump B 2.0 M $(NH_4)_2HPO_4$; 0% B for 5 min to 22% B with a duration of 60 min and then to 100% B over 75 min). Radioactivity eluting from the HPLC column was measured with an on-line continuous flow scintillation detector (Ramona Star). ATP and ADP were

added to the mixture as internal standards and the retention time was determined by UV detection.

2.9. Spectrophotometric detection of RB2

Cells were grown in serum-free chemically defined medium on 60 cm^2 petri dishes in the presence of RB2 (50 μM). At a density of 10^5 cells/cm^2, cells were washed with 6 mL PBS, scraped, and pelleted at 400 *g*. The pellet was suspended and the volume adjusted to 50 μL with 50 mM Tris–HCl (pH 7.4). Subsequently, the cell suspension was boiled for 5 min to denature the proteins and was incubated with 5 U DNAse for 1 hr at 37°. Subsequently, 20 μg proteinase K (Promega) was added and the mixture incubated for 2 hr at 55°. The lysate was centrifuged at 12,000 *g* for 10 min and the amount of RB2 was quantified in the supernatant by measurement of the absorbance at 595 nm in a microplate reader (Benchmark, Bio-Rad).

2.10. Integrity and cellular localization of RB2

The integrity of RB2 was measured by TLC on silica gel using a mixture of *n*-butanol/ethyl acetate/2-propanol/water (20:10:40:30, v/v/v/v). RB2 was dissociated from proteins by denaturation with 0.035 M SDS [25]. Cellular localization of RB2 was determined by equilibrium density centrifugation. C6 cells were rinsed twice with MBS [25 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5), 0.15 M NaCl]. Subsequently, cells were scraped and suspended in MBS containing 1 mM PMSF and were homogenized in a Potter-Elvejhem homogenizer, followed by a 1-min sonication. The sample was mixed with an equal volume of 80% (w/v) sucrose in MBS, applied at the bottom of a discontinuous gradient, and overlaid with 4 mL 30% (w/v) and 4 mL 5% (w/v) sucrose in MBS. Centrifugation was for 22 hr at 100,000 *g*. Twelve fractions (1 mL) were collected and the amount of RB2 quantified by measurement of the absorbance at 595 nm.

2.11. Statistical analysis

Results are represented as the means ± SEM calculated from at least three independent experiments. Statistically significant differences were calculated using the Student's *t* test.

3. Results

We previously reported that stimulation of the $P2Y_{12}$ receptor with 2MeSADP activates mitogen-activated protein kinase (MAPK) and enhanced the proliferation of C6 cells more than 2-fold [26]. Furthermore, stimulation of the latter receptor also inhibited the β-adrenergic receptor-mediated activation of adenylate cyclase, expression of GFAP, and induction of differentiation [13,27]. In this communication, we demonstrated that non-selective P2Y

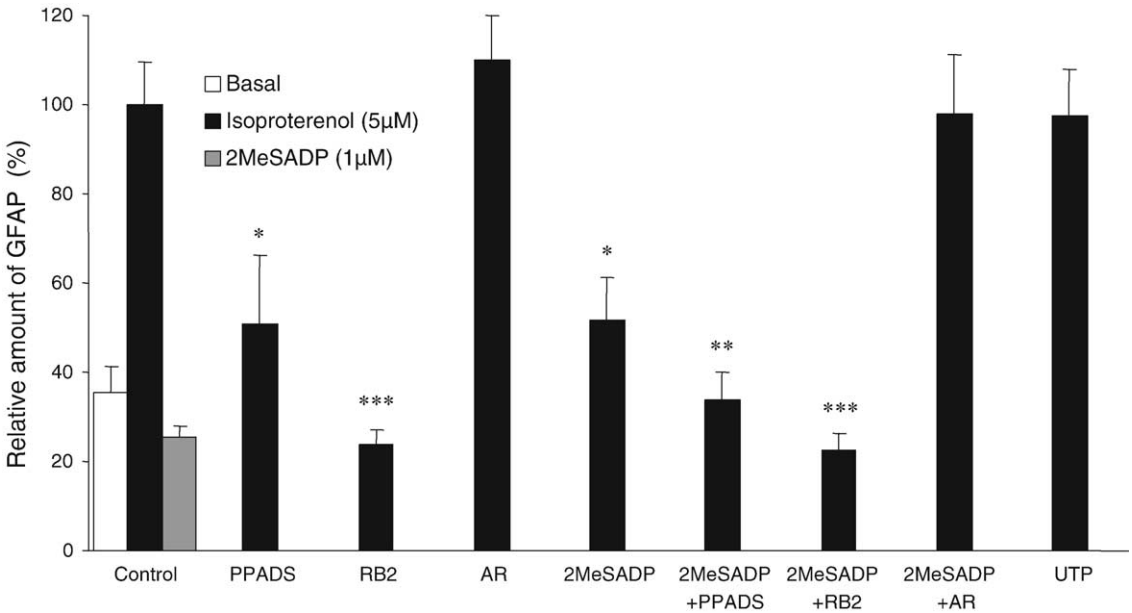

Fig. 1. Inhibition of isoproterenol-induced GFAP synthesis by $P2Y_{12}$ receptor activation. C6 cells were cultivated in 96-well plates in serum-free chemically defined medium up to a density of approximately 0.8×10^5 to 1.2×10^5 cells/cm^2. Fifteen minutes before stimulation with 5 μM (−)-isoproterenol or PBS (basal), the P2Y receptor antagonists [RB2, PPADS, suramin (50 μM), or AR-C69931MX (AR, 10 μM)] or PBS (control) were added. Five minutes before stimulation with 5 μM (−)-isoproterenol, 2MeSADP (1 μM), UTP (100 μM), or PBS were added. After 48-hr incubation, the medium was removed and the cells were analyzed for GFAP by immunoblotting. The amount of GFAP induced by (−)-isoproterenol is taken as 100% (N = 3). The statistically significant difference from the GFAP content of (−)-isoproterenol-stimulated cells is indicated ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$).

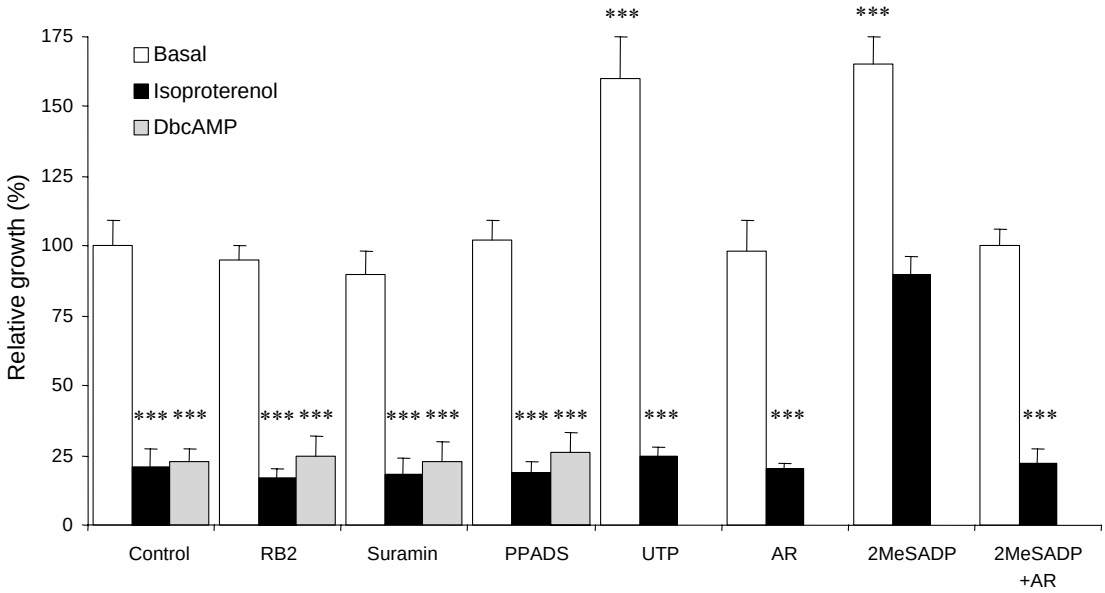

Fig. 2. Effect of $P2Y_{12}$ receptor activation and P2Y antagonists on (−)-isoproterenol- and dbcAMP-induced growth inhibition. C6 cells were cultivated in 96-well plates in serum-free chemically defined medium up to a density of approximately 0.8×10^5 to 1.2×10^5 cells/cm^2. Fifteen minutes before stimulation with 5 μM (−)-isoproterenol, 1 mM dbcAMP or PBS (control), the P2Y receptor antagonists [RB2, PPADS, suramin (50 μM), or AR-C69931MX (AR, 10 μM)] or PBS (basal) were added. Five minutes before stimulation with 5 μM (−)-isoproterenol, 2MeSADP (1 μM) or PBS were added. After 48 hr of cultivation, cell numbers were counted in a hemocytometer. The number of non-stimulated cells (control) is taken as 100% (N = 3). The statistically significant difference from the growth of control cells is indicated ($^{***}P < 0.001$).

receptor antagonists are able to inhibit the β-adrenergic receptor-induced expression of GFAP by a receptor-independent inactivation of PI 3-K.

Stimulation of the β-adrenergic receptor of C6 cells with 5 μM (−)-isoproterenol increased GFAP content and inhibited cell growth more than 2.5-fold (Fig. 1) and 4-fold (Fig. 2), respectively. However, if cells were incubated for 15 min with 1 μM 2MeSADP before addition of (−)-isoproterenol, GFAP synthesis (Fig. 1), and cell growth (Fig. 2) were almost the same as in non-stimulated cells. Differentiation, characterized by growth inhibition, and GFAP synthesis was abolished by activation of the $P2Y_{12}$ receptor and was confirmed by experiments in the presence of the $P2Y_{12}$ receptor antagonist AR-C69931MX. On the other hand, 2MeSADP slightly decreased the basal level of GFAP and decreased dbcAMP-induced GFAP synthesis by less than 30% (Fig. 3). In addition, we also tested UTP, an agonist of the $P2Y_2$ receptor coupled to MAPK activation by a phospholipase C-dependent pathway [26,28]. As expected UTP did not affect GFAP synthesis or growth inhibition induced by activated β-adrenergic receptors (Figs. 1 and 2). The effect of AR-C69931MX was compared with the effect of the non-selective P2Y receptor antagonist RB2. As a negative control, we also incubated cells with PPADS, an antagonist not affecting the $P2Y_{12}$ receptor [29]. Unexpectedly, the $P2Y_{12}$ receptor-mediated inhibition of GFAP expression is not blocked by RB2 (Fig. 1). On the contrary, $P2Y_{12}$ receptor antagonists reduced the basal as well as the (−)-isoproterenol-induced GFAP expression to a different extent (Fig. 3). The (−)-isoproterenol-induced as well as the dbcAMP-induced GFAP expression was inhibited by RB2 and to a lesser extent by suramin and PPADS (Fig. 3). However, none of the antagonists were able to neutralize the growth inhibition that accompanied the cAMP-dependent induction of differentiation (Fig. 2). dbcAMP is a hydrophobic cAMP analogue that has to pass the cell membrane to increase the intracellular cAMP concentration [15]. Therefore, inhibition of dbcAMP-induced GFAP synthesis suggests that nucleotide receptor antagonists also have to enter the cell before they can act as inhibitors of differentiation.

Previously, Roymans *et al*. [16] reported that PI 3-K inhibitors wortmannin and LY294002 abolished GFAP expression after induction of differentiation and caused a pericentrosomal localization of the remaining GFAP. Using CLSM, we analyzed the cellular distribution of GFAP after induction of differentiation in the presence of the P2Y receptor antagonists RB2, PPADS, and suramin (Fig. 4). The GFAP content was decreased to the level in non-stimulated cells and the remaining GFAP showed a perinuclear distribution similar to that observed after induction of differentiation in the presence of wortmannin or LY294002 [16].

These data point to PI 3-K as a potential target enzyme inhibited by these antagonists. Indeed, P2Y antagonists added extracellulary to C6 cells and incubated for 48 hr, or added directly to the PI 3-K activity assay mixture, were able to inhibit PI 3-K to a different extent (Fig. 5A). These results indicated that RB2 and suramin were more efficient than PPADS. However, when cells were incubated with RB2 and suramin for only 15 min, the PI 3-K activity was much less affected, suggesting that receptor antagonists

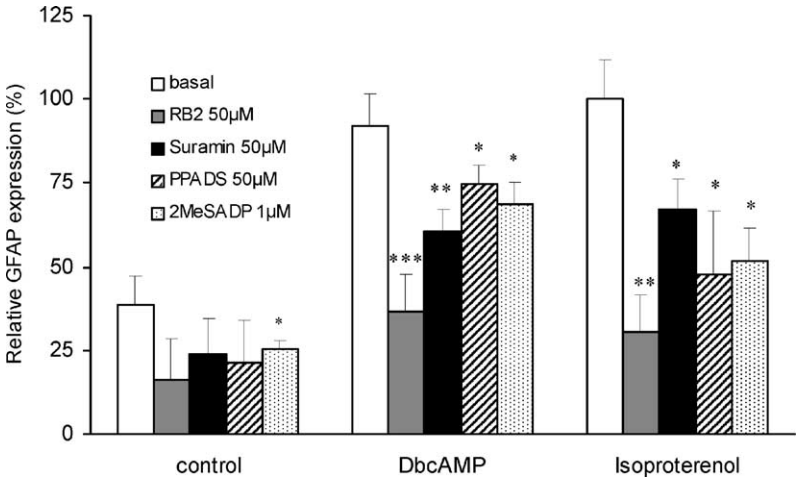

Fig. 3. Inhibition of (−)-isoproterenol- and dbcAMP-induced GFAP synthesis by $P2Y_{12}$ receptor activation and P2Y receptor antagonists. C6 cells were cultivated in 96-well plates in serum-free chemically defined medium up to a density of approximately 0.8×10^5 to 1.2×10^5 cells/cm^2. Fifteen minutes before stimulation with (−)-isoproterenol (5 μM), dbcAMP (1 mM), 2MeSADP (1 μM), or PBS, the P2Y receptor antagonists [RB2, PPADS, suramin (50 μM)] or PBS were added. After 48-hr incubation, the medium was removed and the cells were analyzed for GFAP expression. Cells were dissolved in SDS–PAGE buffer and the GFAP content measured by immunoblotting. The amount of GFAP induced by (−)-isoproterenol is taken as 100% (N = 3). The statistically significant difference from the GFAP content of the respective controls (white bars of control, dbcAMP-, or (−)-isoproterenol-stimulated cells) is indicated ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$).

have to be taken up by the cells (Fig. 5A). Immunodetection of total PI 3-K did not show significant changes in PI 3-K content in the different conditions (data not shown), indicating that the P2Y receptor antagonists did not affect PI 3-K expression. For *in vitro* PI 3-K inhibition an IC_{50} of 4 ± 5 μM was calculated for RB2 (Fig. 5B) while suramin and PPADS are less efficient with an IC_{50} above 50 μM (Fig. 5A).

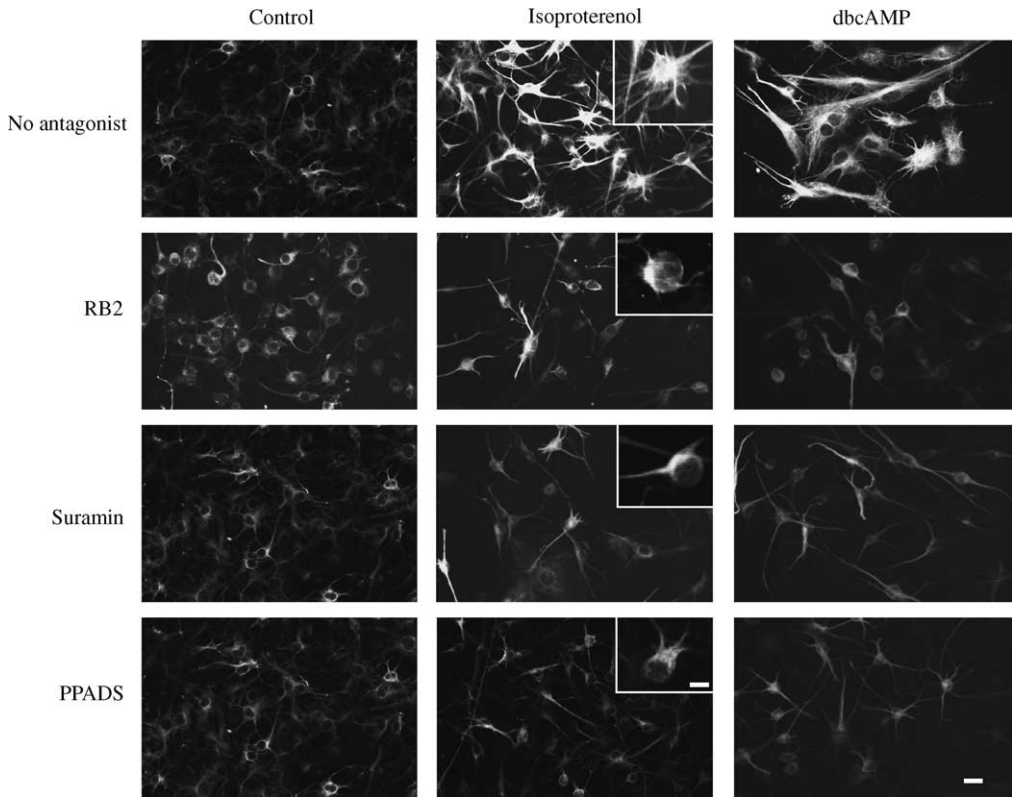

Fig. 4. Effect of P2Y receptor antagonists on the expression and cellular localization of isoproterenol- and dbcAMP-induced GFAP. Cells were grown on coverslips in serum-free chemically defined medium up to a density of approximately 0.8×10^5 to 1.2×10^5 cells/cm^2. Fifteen minutes before stimulation with (−)-isoproterenol (5 μM) or dbcAMP (1 mM), PBS (no antagonist) or P2Y receptor antagonists [RB2, PPADS, suramin (50 μM)] were added. Forty-eight hours after stimulation cells were fixed, stained for GFAP, and the immunofluorescence visualized by CLSM. The inserts in the panels of (−)-isoproterenol-stimulated cells show an enlarged view of the immunofluorescence of GFAP. Bar is 10 μm; for inserts, bar is 20 μm.

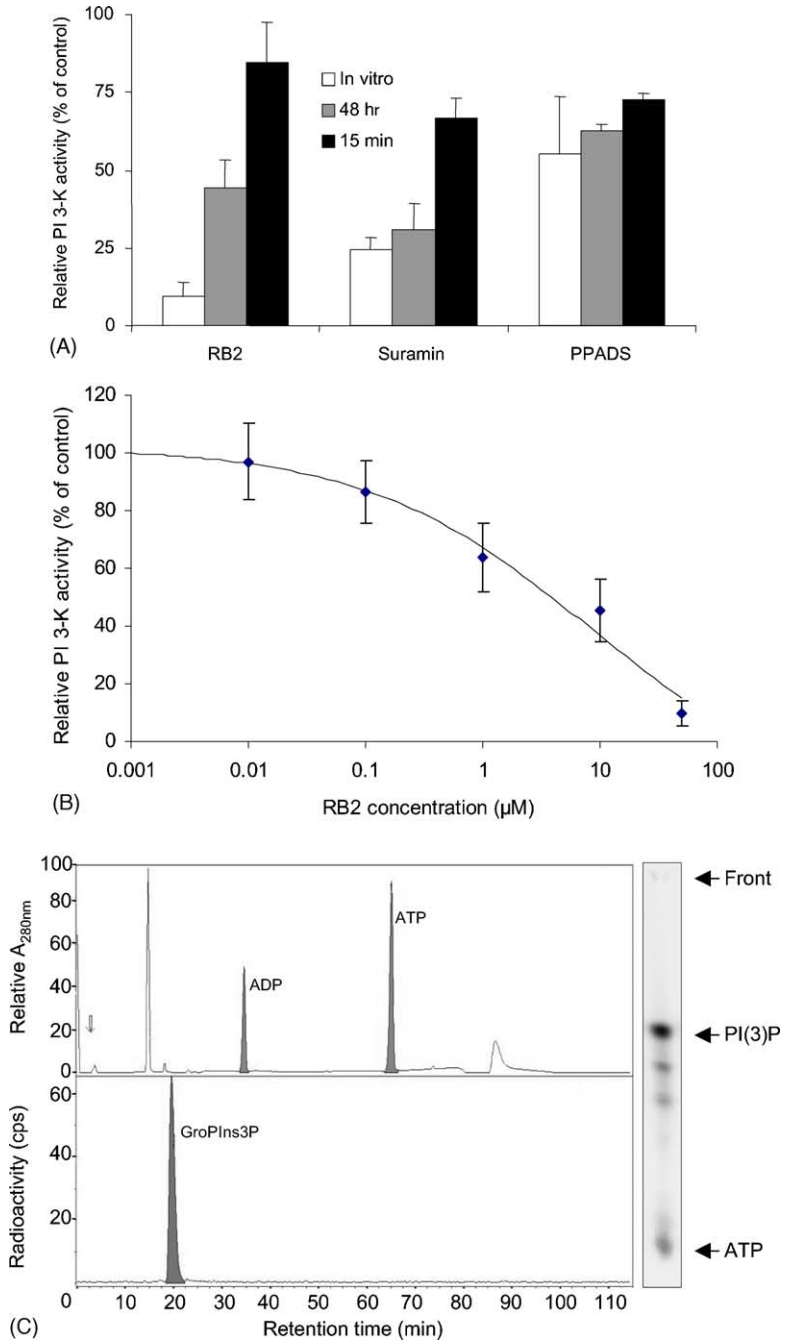

Fig. 5. PI 3-K inhibition by P2Y receptor antagonists. Cells were grown in serum-free chemically defined medium on 60 cm^2 petri dishes. (A) Cells incubated for 15 min or 48 hr with P2Y receptor antagonists (50 μM) were lysed at a density of 1.5×10^4 cells/cm^2. PI 3-K was immunoprecipitated as described in Section 2, and its activity determined. The *in vitro* effect of the antagonists (50 μM) was determined using partially purified PI 3-K from non-stimulated cells. Data (N = 3) are expressed relative to the PI 3-K activity of control cells. (B) Varying concentrations of RB2 were added to the *in vitro* reaction mixture containing PI 3-K partially purified from non-stimulated cells. Data (N = 3) are expressed relative to the PI 3-K activity measured in the absence of RB2. (C) The TLC image (right panel) is representative for three independent experiments. The indicated spot was identified as PI(3)P by HPLC analysis (left panel). To calibrate the HPLC system ATP and ADP were added to the mixture as internal standards before injection and measured by UV absorption in parallel with the on-line radioactivity.

Cellular uptake of RB2 was determined by measurement of the increase in absorbance of cell lysates at 595 nm as a function of time (Fig. 6). To avoid loss of RB2 by precipitation due to binding to macromolecular structures, cells were heat denatured and treated with proteinase K and DNase. After centrifugation RB2 was mostly located in the supernatant and its amount was calculated from the linear concentration/$A_{595\ nm}$ relationship. From a cellular volume of 1.6 μL/10^6 cells [30], the estimated intracellular RB2 concentration increased to approximately 220 μM after 48 hr. To exclude the possibility that inhibition of PI 3-K was due to degradation products of RB2, its integrity was measured by TLC. The same R_f value of 0.61 ± 0.03 was determined for control RB2, extracellular RB2 and RB2

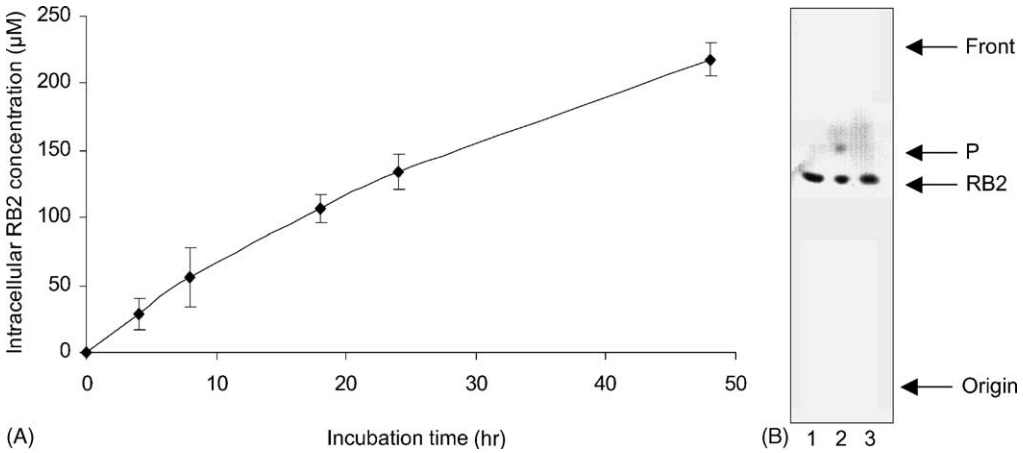

Fig. 6. Cellular uptake and integrity of RB2. Cells were grown in serum-free chemically defined medium on 60 cm^2 petri dishes. (A) After incubation with RB2 (50 μM) for the indicated times the intracellular amount was quantified by measurement of the absorption at 595 nm as described in Section 2. The amount of RB2 was calculated from the linear concentration/$A_{595\ nm}$ relationship. (B) C6 cells were incubated with RB2 (50 μM) for 48 hr and the integrity of RB2 was measured by TLC. Lane 1: control RB2, lane 2: RB2 from the extracellular medium, lane 3: RB2 dissociated from intracellular macromolecular structures by SDS. P: phenol red.

taken up by the cells and released from macromolecular structures by SDS (Fig. 6B). A yellow colored spot with R_f of 0.69 ± 0.01 is phenol red present in the cell cultivation medium. The intracellular localization of RB2 was evaluated by density gradient centrifugation of a lysate of cells incubated for 48 hr with RB2. The lysate was loaded at the bottom of the gradient. At equilibrium approximately 50% of RB2 banded at a density of the membrane fraction (1.17–1.18 g/cm^3) while approximately 50% remained at the bottom of the centrifuge tube ($\rho > 1.2$ g/cm^3) associated with the cytosolic, nuclear, or microsomal fraction.

4. Discussion

Although induction of differentiation and concomitant expression of GFAP in rat C6 glioma cells is known for several years, the regulatory mechanisms are still largely unknown. It has been shown that the cAMP-dependent induction of GFAP expression requires PI 3-K [16] and is independent of PKA activity [15]. In this communication, we describe the effect of $P2Y_{12}$ receptor stimulation on the cAMP-dependent induction of differentiation in C6 cells. Activation of this receptor, negatively coupled to adenylate cyclase, inhibited the (−)-isoproterenol-induced differentiation towards an astrocyte type II. Since stimulation of the $P2Y_{12}$ receptor by 2MeSADP is coupled to MAPK p42/44 [22,26] and PKB activation [27], and PKB is inhibited in a cAMP-dependent manner, it has been proposed that agonists of the $P2Y_{12}$ receptor reverse the isoproterenol-induced differentiation by inhibition of cAMP synthesis concomitant with reactivation of PKB [27]. dbcAMP-induced differentiation bypasses adenylate cyclase and is only slightly affected by $P2Y_{12}$ receptor stimulation. The small but significant decrease in dbcAMP-induced GFAP synthesis upon $P2Y_{12}$ receptor stimulation may be explained by decrease of the basal level of GFAP although it cannot be excluded that beside inhibition of adenylate cyclase other intracellular targets are involved in the $P2Y_{12}$-mediated effect on GFAP synthesis. To unequivocally demonstrate that activation of MAPK is not involved in the reduced GFAP expression, we compared the effect of $P2Y_{12}$ and $P2Y_2$ receptor activation. The latter receptor is stimulated by UTP and is coupled to phospholipase C and MAPK activation, but not to adenylate cyclase [26,28]. UTP added prior to stimulation of the cells with (−)-isoproterenol had no effect on GFAP expression, clearly demonstrating necessity of an inhibition of adenylate cyclase but not of phospholipase C and MAPK activation. Furthermore, antagonizing the $P2Y_{12}$ receptor with AR-C69931MX counteracted its activation by 2MeSADP. We also analyzed the effect of RB2, a P2Y receptor antagonist that inhibits almost all P2Y receptors including the $P2Y_{12}$ receptor, and PPADS, an antagonist that blocks several P2Y receptors but not the $P2Y_{12}$, on the $P2Y_{12}$ receptor-mediated inhibition of GFAP expression. In contrast to AR-C69931MX, RB2 did not neutralize the receptor-induced inhibition of differentiation although it has been shown to revert the $P2Y_{12}$-mediated inhibition of adenylate cyclase [22]. Notwithstanding, the different antagonist profiles towards P2Y receptors, both RB2 and PPADS inhibited the (−)-isoproterenol-induced synthesis of GFAP, indicating that this effect is not due to their antagonistic action on nucleotide receptors. Furthermore, suramin a non-specific antagonist of P2Y receptors was also able to inhibit the (−)-isoproterenol-induced GFAP expression. In addition, none of the antagonists had an effect on the (−)-isoproterenol-induced growth inhibition. All three antagonists also blocked the dbcAMP-induced GFAP expression. dbcAMP is a membrane-permeable cAMP analogue that has to pass the plasma membrane before it is hydrolyzed into cAMP by cellular esterases and

can activate intracellular signal transduction pathways. This was a first indication that extracellularly added P2Y receptor antagonists had to enter the cell before they can inhibit intracellular targets involved in the induction of GFAP synthesis and that the observed effect is not mediated by an antagonizing effect on the $P2Y_{12}$ receptor.

PI 3-K has been identified as a necessary factor for GFAP expression and therefore assumed to be a potential intracellular target for inhibition by P2Y receptor antagonists. Roymans *et al*. [16] reported that addition of the PI 3-K inhibitors wortmannin or LY294002, or the expression of dominant negative PI 3-K mutants blocked GFAP expression upon cAMP-dependent induction of differentiation. The remaining GFAP is localized in the pericentrosomal region of the cell. Furthermore, inhibition of PI 3-K results in an altered cell shape lacking process formation specific for the astrocytic differentiation stage. CLSM of C6 cells preincubated with the P2Y receptor antagonists RB2, PPADS, and suramin indicated that these compounds inhibited cAMP-induced GFAP expression to a different extent and that the cells form less processes.

Enzyme assays further demonstrated that PI 3-K was an intracellular target of RB2 and suramin. In agreement with previous data, the enzyme is constitutively active in C6 cells [16]. The P2Y receptor antagonists RB2 and suramin inhibit the purified enzyme '*in vitro*'. Furthermore, the cellular PI 3-K activity was also inhibited after 48-hr incubation of the cells with these purinergic receptor antagonists. However, when the cells were incubated for only 15 min, PI 3-K activity was less affected. Since the action of antagonists on receptor activation is almost immediate, these data demonstrate that inhibition of PI 3-K requires cellular uptake of these compounds. Since these compounds are large heterocyclic molecules, one can assume that they are membrane permeable. However, these P2Y receptor antagonists contain charged sulfate groups, and it can be questioned whether they enter the cell by migration through the cell membrane. In human microvascular endothelial cells suramin uptake involves the caveolae system and is primarily located in the cytoplasmic or nuclear fraction, depending on the incubation time [31]. In human colon adenocarcinoma cells quantitative autoradiography localized suramin in the nucleus, the Golgi apparatus, and the mitochondria [32]. In the presence of serum albumin uptake of suramin was strongly reduced in endothelial cells and localized in the lysosomal compartment of adenocarcinoma cells [31,32]. Up to now, cellular uptake of PPADS and RB2 has not been studied and it remains to be determined if caveolae are also involved. Although we reported that the $P2Y_{12}$ receptor present on C6 cells is completely desensitized within 4 hr [22], we may exclude the possibility that receptor antagonists are internalized by downregulation of P2Y receptors [33–35]. Indeed, RB2 uptake experiments were performed in the absence of $P2Y_{12}$ receptor activation where internalization should be relatively insignificant. Although the uptake of RB2 was clearly proven by the gradual increase in absorbance of the cell lysate at 595 nm with increasing incubation time, the uptake mechanism remains to be determined. We calculated that RB2 accumulates in the cell up to concentrations exceeding the added extracellular concentration. In addition, we observed that approximately half of the intracellular RB2 was bound to membranes while the other half was distributed over the cytoplasmic, nuclear, or microsomal fraction.

Although a function of PI 3-K in cAMP-induced GFAP expression is demonstrated [16], it is not unlikely that other unidentified factors involved in GFAP expression become inhibited by RB2. Inhibition of GFAP synthesis by PPADS, a weak *in vitro* inhibitor of PI 3-K, also points to the possible involvement of additional factors.

Although GFAP synthesis was inhibited, the growth inhibition that parallels the induction of differentiation was not abolished by incubation of C6 cells with purinergic receptor antagonists. C6 cells were still growth arrested after co-stimulation with (−)-isoproterenol or dbcAMP and receptor antagonists, although GFAP expression was blocked. This suggests that growth inhibition and GFAP expression proceed through different pathways and that the signal transduction pathway leading to growth arrest upon induction of differentiation is independent of PI 3-K activity.

The presented results are of importance for the interpretation of data obtained with the P2Y receptor antagonist RB2. Since a significant amount of this compound is taken up by the cells after incubation times exceeding 30 min, care must be taken upon its use in experiments with long incubation times.

In this communication, we presented evidence that cAMP-induced differentiation is inhibited by a nucleotide-mediated mechanism involving activation of the $P2Y_{12}$ receptor. In addition, the expression of GFAP, a differentiation marker of astrocytes, is also inhibited by cellular uptake of nucleotide receptor antagonists that inhibit PI 3-K or other unidentified factors required for the induction of GFAP synthesis.

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