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A phosphatidylinositol transfer protein α-dependent survival factor protects cultured primary neurons against serum deprivation-induced cell death

Hanneke Bunte,*,† Martijn Schenning,* Peter Sodaar,† Dop P. R. Bär,‡ Karel W. A. Wirtz,* Freek L. van Muiswinkel†,[1] and Gerry T. Snoek*,[1]

*Bijvoet Center for Biomolecular Research, Department of Biochemistry of Lipids, Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands

†Department of Neurology, Rudolf Magnus Institute of Neuroscience, University Medical Centre Utrecht, Utrecht, The Netherlands

‡Graduate School for Biomedical Sciences, University Medical Centre Utrecht, Utrecht, The Netherlands

Abstract

Selective neuronal loss is a prominent feature in both acute and chronic neurological disorders. Recently, a link between neurodegeneration and a deficiency in the lipid transport protein phosphatidylinositol transfer protein α (PI-TPα) has been demonstrated. In this context it may be of importance that fibroblasts overexpressing PI-TPα are known to produce and secrete bioactive survival factors that protect fibroblasts against UV-induced apoptosis. In the present study it was investigated whether the conditioned medium of cells overexpressing PI-TPα (CMα) has neuroprotective effects on primary neurons in culture. We show that CMα is capable of protecting primary, spinal cord-derived motor neurons from serum deprivation-induced cell death. Since the conditioned medium of wild-type cells was much less effective, we infer that the neuroprotective effect of CMα is linked (in part) to the PI-TPα-dependent production of arachidonic acid metabolites. The neuroprotective activity of CMα is partly inhibited by suramin, a broad-spectrum antagonist of G-protein coupled receptors. Western blot analysis shows that brain cortex and spinal cord express relatively high levels of PI-TPα, suggesting that the survival factor may be produced in neuronal tissue. We propose that the bioactive survival factor is implicated in neuronal survival. If so, PI-TPα could be a promising target to be evaluated in studies on the prevention and treatment of neurological disorders.

Keywords: motor neurons, neurodegeneration, neuroprotection, phosphatidylinositol transfer protein α, phospholipid transport protein.

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Neurodegenerative disorders are marked by selective and progressive neuronal loss with consequent neurological deficits. Insight into the mechanisms controlling neuronal survival and death may provide important clues regarding the development of neuroprotective strategies. Interestingly (motor) neuron degeneration has recently been linked to the reduced expression of phosphatidylinositol transfer protein α (PI-TPα). Thus, the so-called vibrator mouse, which suffers from progressive action tremor caused by degeneration of motor neurons in the brainstem and spinal cord, showed a reduction in brain PI-TPα expression (Hamilton *et al*. 1997). Moreover, PI-TPα knock-out mice die within 14 days after birth due to progressive spinocerebellar degeneration (Alb *et al*. 2003). On the other hand, by using a NIH3T3 mouse fibroblast cell line overexpressing PI-

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Address correspondence and reprint requests to Hanneke Bunte, MSc, Bijvoet Center for Biomolecular Research, Department of Biochemistry of Lipids, Institute of Biomembranes, Utrecht University, PO Box 80054, 3508 TB Utrecht, The Netherlands.
E-mail: h.bunte@chem.uu.nl

[1]These authors contributed equally to this study.

Abbreviations used: ALS, amyotrophic lateral sclerosis; CB1R, cannabinoid 1 receptor; CMα/wt, conditioned medium of SPIα cells/wild-type NIH3T3 fibroblasts; COX-2, cyclooxygenase-2; ECE, embryonic chicken extract; GPCR, G-protein coupled receptor; MN, motor neuron; NLEα/wt, neutral lipid extract of CMα/wt, containing arachidonic acid metabolites; PI-TPα, phosphatidylinositol transfer protein α; PLA_2, phospholipase A_2; SPIα cells, NIH3T3 mouse fibroblasts overexpressing P_1-TPα.

TPα, it has been shown that the overexpression of PI-TPα is coupled to enhanced cellular protection (Schenning *et al*. 2004).

PI-TPα belongs to the family of lipid transport proteins and is highly conserved throughout mammalian evolution. At least *in vitro* it has been shown that PI-TPα binds and transfers phosphatidylinositol and phosphatidylcholine between membranes (Helmkamp *et al*. 1974). In mice, the highest abundance of PI-TPα has been reported to occur in brain (van Tiel *et al*. 2004).

Analysis of the inositol metabolites in PI-TPα-overexpressing cells indicated the activation of a PI-specific phospholipase A_2 (PLA_2), resulting in an enhanced production of lysophosphatidylinositol and other degradation products such as arachidonic acid and its metabolites (Snoek *et al*. 1999; Schenning *et al*. 2004).

Interestingly, conditioned medium from PI-TPα-overexpressing cells (CMα) has been demonstrated to stimulate the rate of proliferation of wild-type (wt) NIH3T3 cells and to confer protection against UV radiation and TNFα treatment. This indicates that PI-TPα overexpressing cells secrete both PI-TPα-dependent mitogenic and survival factors. Since the production and metabolism of arachidonic acid is increased in the PI-TPα overexpressing fibroblasts, the protective property of CMα may be attributable, at least in part, to arachidonic acid derived compounds (Schenning *et al*. 2004). This is corroborated by the observations that (i) an arachidonic acid enriched lipid extract of CMα expresses a protective activity similar to that of CMα and (ii) inhibition of cyclooxygenase-2 (COX-2) reduces the anti-apoptotic efficacy of CMα.

Given the relationship between PI-TPα expression and neurodegeneration in transgenic mouse models, and the profound anti-apoptotic efficacy of CMα, we hypothesize that PI-TPα may be critically involved in the production of a survival factor that is implicated in neuronal survival. To test this hypothesis, we have investigated and characterized the effect of CMα on the survival of serum-deprived neurons, using spinal cord-derived, rat motor neurons as a model.

Materials and methods

Chemicals

Trypsine (Calbiochem, San Diego, CA, USA), laminine (Life Technologies, Grand Island, NY, USA), culture media and sera (Gibco, Rockville, MD, USA), glycin (J. T. Baker, Phillipsburg, NJ, USA), goat anti-rabbit horseradish peroxidase antibody (Bio-Rad, Hercules, CA, USA), mowiol 4-88 (Calbiochem) and enhanced chemilumincence detection reagents [Amersham Biosciences, Piscataway, NJ, USA (Now GE Healthcare Bio-sciences corp.)] were obtained from the suppliers indicated. SR141716A was a gift from Dr G. van Zadelhoff (Section Bio-organic Chemistry, Bijvoet Centre, Utrecht University). Unless mentioned otherwise, all other chemicals were obtained from Sigma (St Louis, MO, USA).

Primary motor neuron culture

Motor neurons were isolated from E15 Wistar rat embryonic spinal cord by metrizamide density centrifugation as previously described (Camu and Henderson 1992; Kaal *et al*. 1997). In brief, the ventral part of the spinal cord of three embryos was dissected and cut into small pieces before being trypsinized (0.05% w/v) for 15 min at 37°C. After degrading liberated DNA with 100 μg/mL DNAse I, the cell suspension was put on a 6.5% metrizamide cushion and centrifuged for 15 min at 790 ***g***. Cells from the interphase were collected, put on a 4% bovine serum albumin cushion and centrifuged at 170 *g* for 10 min. Finally, the pellet was resuspended in motor neuron culture medium and plated in polyornithine (1.5 μg/mL) and laminine (3 μg/mL) coated 24-well plates at a density of 12 000 cells per well. Cells were grown at 37°C, 6% O_2 and 5% CO_2 in a humified atmosphere.

Motor neuron culture medium (MN medium) consisted of L15 medium containing $NaHCO_3$ (22 mM), insulin (5 μg/mL), putrescine (0.1 mM), conalbumin (0.1 mg/mL), sodium selenite (30 nM), glucose (20 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and embryonic chicken muscle extract (ECE, 1.2%: Henderson *et al*. 1983).

Primary astrocyte culture

To obtain primary astrocyte cultures, the pellet fraction obtained after the metrizamide step in the motor neuron purification procedure was resuspended in culture medium. Cells were plated in polyornithine (1.5 μg/mL)-coated 75-cm^2 culture flasks and grown to confluence at 37°C and 5% CO_2. Subsequently, non-astrocytic contaminating cells were shaken off on a rotary shaker (250 r.p.m.; overnight; 37°C) (Giulian and Baker 1986). Culture medium was L15 medium containing $NaHCO_3$ (22 mM), glucose (20 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and fetal bovine serum (10%).

Preparation of conditioned media and neutral lipid extracts

Conditioned media (CM) were prepared as described previously (Schenning *et al*. 2004). Briefly, cell cultures of either wild-type NIH3T3 mouse fibroblasts or NIH3T3 cells overexpressing PI-TPα (SPIα cells) were grown to 80–90% confluence in Dulbecco's modified Eagle's medium containing 10% newborn calf serum before the medium was replaced by ECE-free MN medium containing 0.1% bovine serum albumin. After 24 h, the medium was collected and centrifuged (5 min at 500 *g*) to yield either CMα or CMwt (from SPIα or wtNIH3T3 cells, respectively). Conditioned media were stored at 4°C until further use.

Neutral lipid extracts (NLEs) containing arachidonic acid metabolites, were obtained from CMα and CMwt as described previously (Tai *et al*. 1976). In short, upon addition of 0.03 mL of 12 M formic acid to 1 mL of CM, the mixture was extracted with two 3 mL volumes of ethyl acetate. The combined extracts were then evaporated under nitrogen before the residues were dissolved in ethanol to yield NLEα and NLEwt, respectively, which were immediately used after preparation.

Experimental design

The effect of CM and NLE on the survival of motor neurons upon serum deprivation was monitored as a function of time. Therefore a healthy subpopulation of motor neurons (± 25 neurons/well) was

identified 4 days after plating by phase contrast microscopy and mapped with a Leica DM IRBE microscope (Rijswijk, the Netherlands) equipped with the Leica Quantimet images analysis system software. Healthy motor neurons were defined as phase bright cells containing neurites longer than two cell diameters (Camu and Henderson 1992; Kaal *et al.* 1997; Wisman *et al.* 2003). Serum deprivation-induced cell death was induced by replacing the medium with ECE-free MN medium. The effect of CMα, CMwt and the respective NLEs on survival was assessed by supplementing the ECE-free MN medium with various dilutions of these media. When motor neurons growing on 1 cm^2 of well surface were incubated with CM from 1 cm^2 of confluent fibroblasts, this condition was defined as incubation with undiluted CM (1 : 1). Accordingly, a three times dilution of CM (CM 1 : 3) means that motor neurons growing on 3 cm^2 of surface of were incubated with CM from 1 cm^2 of fibroblasts.

At different time points following the start of serum deprivation, the viability of mapped motor neurons was assessed under phase contrast microscopy using well-established criteria to distinguish between healthy and dead cells (intact cell body and processes vs. fragmented processes and the presence of granule- and vacuole-like structures in the cell body, respectively) (Kaal *et al.* 1998).

When indicated, the effect of the broad-spectrum antagonist of the G-protein coupled receptor family suramin (100 μm) (Chung and Kermode 2005), or the cannabinoid 1 receptor antagonist SR141716A (rimonabant; 10 μm) (Boyd and Fremming 2005) was tested on the CMα-induced neuroprotection as observed within the first 24 h of treatment.

Immunocytochemistry

Primary motor neurons and confluent astrocyte cultures, grown on polyornithine and laminine- or polyornithine-coated glass cover slips, respectively, were washed with phosphate-buffered saline (PBS) (pH 7.4) and fixed in 4% paraformaldehyde in phosphate-buffered saline for 30 min at room temperature. After washing with PBS, the cells were permeabilized in 0.5% Triton X-100 in PBS for 5 min and, after an additional washing step, incubated for 10 min in 50 mM glycine in PBS. Blocking occurred in HEPES-buffered Dulbecco's modified Eagle's medium containing 0.5% bovine serum albumin for 30 min.

PI-TPα immunoreactivity was demonstrated using an anti-PI-TPα polyclonal antibody (1 : 100) (Snoek *et al.* 1992). After 60 min, the cells were washed and incubated with goat anti-rabbit-Cy3 for 60 min. All antibodies were diluted in Dulbecco's modified Eagle's medium–HEPES containing 0.1% bovine serum albumin. Cells were mounted in mowiol 4-88 containing 0.1% 1,4-diazabicyclo[2,2,2]octane. Fluorescence was visualized by confocal laser scanning microscopy using a Nikon Eclipse TE2000-U microscope equipped with a confocal C1 unit. Cy3 was excited with the 543 nm line of a He–Ne laser and a 585/30 emission filter.

Analysis of phosphatidylinositol transfer protein α expression in mouse CNS tissue

Three female C57BL/6J@Rj mice (Jackson Laboratories, Bay Harbour, ME, USA), 90 days of age, were anaesthetized and decapitated. Quickly, the liver, cortex and the whole lumbar part of the spinal cord were collected in ice cold lysis buffer (50 mM Tris-HCl pH 7.4, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM $NaVO_4$; 20 μL/mg tissue). The tissue was homogenized, centrifuged at 10 000 *g* for 10 min before the supernatant was stored at −20°C until use. Aliquots of 20 μg of protein were resolved on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose membrane. 10, 25 and 50 ng of purified PI-TPα were used as a calibration curve. After blocking in non-fat milk powder for 1 h, membranes were incubated with an affinity-purified polyclonal rabbit antibody raised against synthetic peptides representing specific epitopes of PI-TPα (1 : 500) (Snoek *et al*. 1992) for 1 h. After washing, a goat anti-rabbit antibody conjugated to horseradish peroxidase (1 : 5000) was used as a secondary antibody (1 h). Membranes were washed and developed with enhanced chemiluminescence detection reagents according to the manufacturer's instructions.

Statistical analysis

For the multiple comparisons between groups, after testing for the homogeneity of variance and for normality of residuals, an analysis of variance (ANOVA) was performed. p-values < 0.05 were considered statistically significant.

Results

The effect of CMα on serum deprivation-induced motor neuron death

Serum deprivation is a well-known inducer of cell death in neuronal cell lines as well as in primary neuronal cultures (Xu *et al*. 2001). As illustrated in Fig. 1, discrimination between viable and dead motor neurons was made on a morphological basis. Viable motor neurons were defined as cells containing intact cell bodies and processes (Fig. 1a), whereas degenerating, dead cells were distinguished by the presence of fragmented processes and granule- and vacuole-like structures within the cell body (Fig. 1b). It has previously been demonstrated that the discrimination between healthy and dead cells on the basis of phase contrast microscopy strongly correlates with live/death assays (Bär 2000). Table 1 shows that incubation of primary motor neurons with ECE-free MN medium resulted in a marked cell loss of approximately 70% within 96 h. However, when motor neurons were grown in the presence of CMα the survival was greatly increased. As shown, a concentration-dependent neuroprotective effect was observed, with a 1 : 3 dilution of CMα showing optimal protection. Under this condition cell survival increased from 31 ± 4 to 80 ± 3% (mean ± SEM) at 96 h. When tested at higher concentrations (i.e. up to 1 : 1), CMα appeared to be less effective, which may be explained by the presence of some toxic factors in the same medium.

The effect of CMwt on serum deprivation-induced motor neuron death

To investigate whether the overexpression of PI-TPα in SPIα cells is indeed responsible for the neuroprotective activity of CMα, the effect of conditioned medium of wtNIH3T3

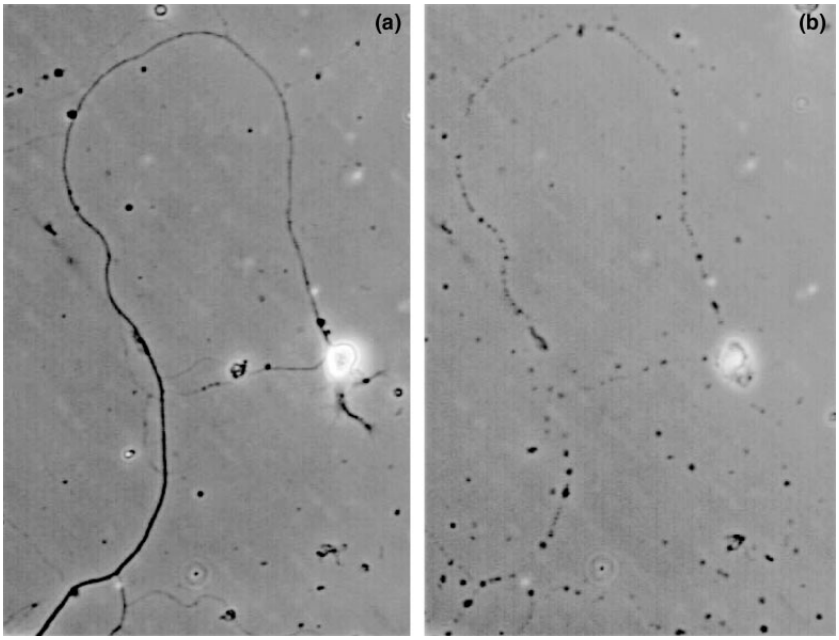

Fig. 1 Phase contrast microscope images showing primary motor neurons in culture. The images show the same neuron before (a) and after (b) 24 h of serum deprivation. Note fragmented processes and the presence of granule and vacuole-like structures in the cell body of the degenerating motor neuron in (b).

Table 1 The neuroprotective effect of conditioned medium of cells overexpressing PI-TPα (CMα) on primary motor neurons

Condition	Survival at 96 h (as percentage of $t = 0$ h)
ECE-free MN medium	31 ± 4
+ CMα 1 : 100	41 ± 11
+ CMα 1 : 30	45 ± 12
+ CMα 1 : 10	67 ± 4*
+ CMα 1 : 3	80 ± 3*
+ CMα 1 : 1	68 ± 9*

After 4 days in culture, to trigger serum deprivation-induced cell death, primary rat motor neurons were incubated in embryonic chicken extract (ECE)-free motor neuron (MN) medium, supplemented with or without conditioned medium from phosphatidylinositol transfer protein α (PI-TP$^{\alpha}$)-overexpressing NIH3T3 mouse fibroblasts (CM$^{\alpha}$). Survival was scored at 24, 48, 72 and 96 h after treatment under phase contrast microscopy using well-defined morphological criteria. Data are mean ± SEM and are representative for at least two independent experiments performed in quadruplicate. *$p < 0.05$ relative to the survival observed in ECE-free MN medium.

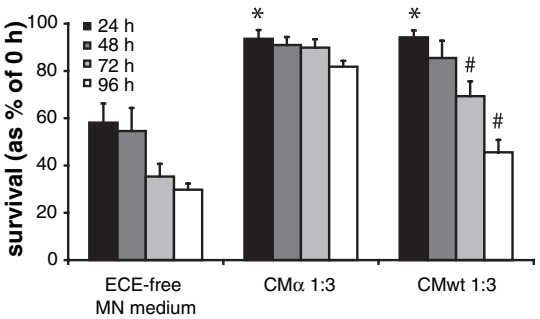

Fig. 2 Conditioned medium of cells overexpressing PI-TPα (CMα), but not conditioned medium of wild-type NIH3T3 fibroblasts (CMwt), protects primary motor neurons against serum deprivation-induced cell death. Four days after plating, motor neurons were incubated in embryonic chicken extract (ECE)-free motor neuron (MN) medium, containing CMα at the optimal concentration (1 : 3) or the same concentration of CMwt. Survival was scored after 24, 48, 72 and 96 h under phase contrast microscopy using well-defined morphological criteria and expressed as percentage of the survival at the start of the treatment. Data are mean ± SEM of eight observations determined in two independent experiments. *$p < 0.05$ relative to the survival in ECE-free MN medium at 24 h #$p < 0.05$ vs. the survival observed in CMα-treated cultures at the same time point.

fibroblasts (CMwt) was tested. As shown in Fig. 2, during the first 24 h of treatment, motor neurons treated with CMwt at a dilution of 1 : 3 also showed increased survival when compared to control cells (94 ± 3 vs. 58 ± 8%, respectively). After 24 h, however, motor neuron loss in the CMwt-treated wells proceeded in a similar way to that observed in the control cells treated with ECE-free MN medium. Thus, the percentage of cell loss in cultures treated with CMwt amounted to 48 ± 4% between 24 and 96 h, which was not statistically different from that observed in control cells (54 ± 7%). At all time points studied, the 1 : 3 dilution of CMα was more effective in enhancing the survival of motor neurons as compared to CMwt.

As wtNIH3T3 fibroblasts do express some endogenous PI-TPα (Snoek *et al*. 1999), CMwt was also tested at higher concentrations, since the amount of survival factor may be

limited in CMwt. However, even at a three times higher concentration, or after renewed addition of CMwt (1 : 3) after 48 h of treatment, CMwt was not able to prevent motor neuron death (data not shown). Taken together, these data indicate that overexpression of PI-TPα is required for the production and secretion of the survival factors involved in preventing motor neuron death.

The effect of a neutral lipid extract of CMα on serum deprivation-induced motor neuron death

Since the production and metabolism of arachidonic acid is increased in SPIα cells (Schenning *et al*. 2004), it was tested whether a neutral lipid extract derived from CMα (NLEα), containing mainly arachidonic acid metabolites, could protect primary motor neurons. As illustrated in Fig. 3, NLEα protected motor neurons almost completely up to a period of 72 h, whereas NLE obtained from CMwt (NLEwt) was virtually inactive. On the basis of the amount of CMα extracted, NLEα was approximately 30 times less efficient than CMα in providing optimal protection. This may indicate either that the neutral lipids including the arachidonic acid metabolites in CMα are only partially responsible for the protective effect or that the active metabolites are rapidly inactivated upon extraction.

The effect of suramin and rimonabant on CMα-induced neuroprotection

In a previous study, evidence was obtained that the survival factor in CMα may act on a cannabinoid 1-like receptor (CB1R) a member of the family of G-protein coupled receptors (GPCR) (Kostenis 2004; Schenning *et al*. 2004).

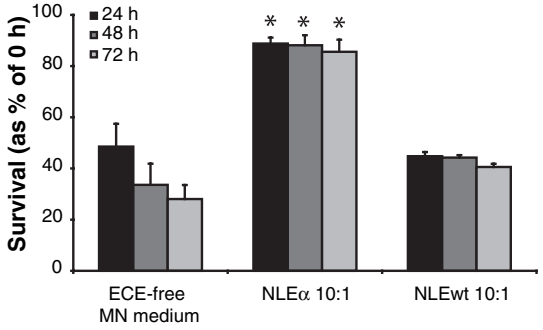

Fig. 3 Survival of primary motor neurons incubated with neutral lipid extracts obtained from conditioned medium of cells overexpressing PI-TPα (CMα) and conditioned medium of wild-type NIH3T3 fibroblasts (CMwt), containing arachidonic acid metabolites (NLEα and NLEwt, respectively). Incubation with embryonic chicken extract (ECE)-free motor neuron (MN) medium served as a control. Survival was scored 24, 48 and 72 h after treatment and expressed as percentage of the survival at the start of the treatment. Data are mean ± SEM from 12 observations determined in three independent experiments. *$p < 0.05$ relative to the survival in ECE-free MN medium at the same time points.

To further characterize the neuroprotective effect of CMα in our neuronal model, the effect of CMα was tested in the presence of suramin, a broad-spectrum non-specific antagonist of GPCRs, and SR141716A (rimonabant, acomplia™), a specific antagonist of the CB1R (Fig. 4). Although suramin (100 μM) itself had no effect on the survival of motor neurons in ECE-free MN medium, co-incubation with this antagonist resulted in a 50% decrease of the neuroprotective effect of CMα (Fig. 4a). Similarly, in the presence of 10 μM rimonabant, a 75% decrease in the CMα-mediated neuroprotective effect could be observed (Fig. 4b). However, at the concentration used, this particular antagonist already caused a 50% decrease in motor neuron viability in the control situation.

Phosphatidylinositol transfer protein α expression in cultured (non-) neuronal cells and mouse CNS

Previously, it has been demonstrated that PI-TPα is most abundant in total brain homogenate (van Tiel *et al*. 2004). In agreement with this, we found that PI-TPα levels in mouse

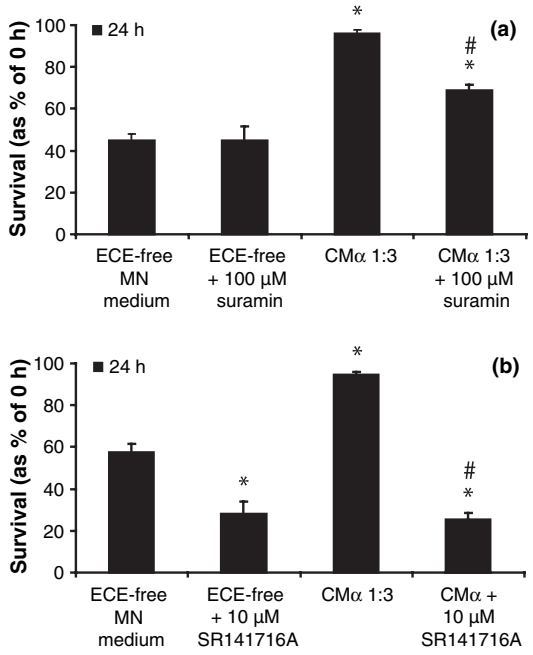

Fig. 4 The role of G-protein coupled receptors in the CMα-mediated effect. Four days after plating, primary motor neurons were treated with the optimal concentration of conditioned medium of cells overexpressing PI-TPα (CMα) with or without (a) 100 μM suramin, a broad-spectrum antagonist of G-protein coupled receptors, or (b) 10 μM SR141716A (rimonabant), a specific antagonist of the cannabinoid 1 receptor. Embryonic chicken extract (ECE)-free motor neuron (MN) medium with or without these compounds served as a control. Survival was scored after 24 h. Data are mean ± SEM from eight observations obtained in two independent experiments. *$p < 0.05$ relative to the survival in ECE-free MN medium. #$p < 0.05$ vs. the survival observed in CMα-treated cultures.

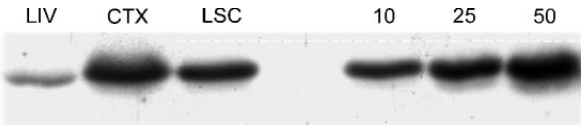

Fig. 5 Expression of phosphatidylinositol transfer protein α (PI-TPα) in tissue. Protein homogenates (20 μg) from female C57BL/6J@Rj mouse liver (LIV), cortex (CTX) and lumbar spinal cord (LSC) tissue were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analysed by Western blot. Coomassie Brilliant Blue staining was exploited to confirm equal loading and 10, 25 and 50 ng of purified PI-TPα was used as a calibration curve.

brain cortex but also in lumbar spinal cord tissues are much higher than the levels detected in the liver (Fig. 5). By using a calibration curve based on purified PI-TPα, and with the conformation of equal protein loading by means of Coomassie Brilliant Blue staining of a parallel-runned gel, it could be estimated that PI-TPα levels in cortex are about twice as high as compared to lumbar spinal cord.

By using a polyclonal antibody, the cellular expression of PI-TPα was investigated in primary spinal cord-derived motor neurons (grown for 4 days in MN medium) and primary astrocyte cultures. As shown in Fig. 6, PI-TPα is markedly expressed in the nuclei of both motor neurons (Fig. 6a) and astrocytes (Fig. 6b). Moreover, besides a moderate expression in the cytosol of both cell types, marked staining was observed in the dendritic and axonal processes of the motor neurons. Overall, this cellular expression pattern is comparable with that previously observed in NIH3T3 fibroblasts (de Vries *et al*. 1995).

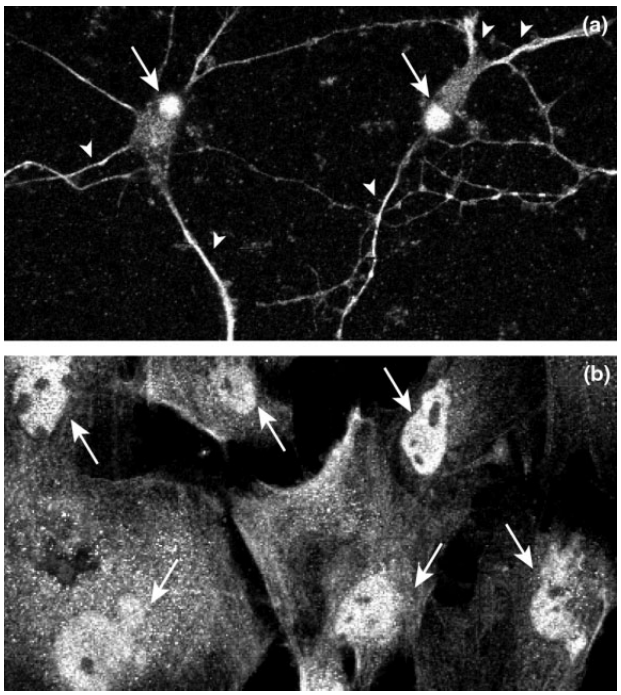

Fig. 6 Confocal laser scanning microscopy images showing the cellular expression of phosphatidylinositol transfer protein α (PI-TPα) in primary motor neurons (a) and embryonic spinal cord astrocytes (b). PI-TPα is predominantly expressed in the nuclei of both cell types (arrows), with moderate expression being observed in the cytosol. Arrowheads indicate the marked staining of PI-TPα in the dendritic and axonal processes of the motor neurons.

Discussion

In this study, we report that the medium conditioned by mouse fibroblasts overexpressing PI-TPα protects primary motor neurons against serum deprivation-induced cell death. Present and previous data indicate that COX-2-derived neutral lipids (including endocannabinoids) secreted into the conditioned medium are partially responsible for this effect (Schenning *et al*. 2004). Moreover, we found that part of the protective effect was inhibited by a broad-spectrum antagonist of GPCRs, indicating that this type of receptor is involved in the protective mechanism.

A direct link between PI-TPα and neurodegeneration first became apparent from the observation that mice homogenous for the so-called vibrator mutation develop action tremor, brainstem and spinal cord degeneration and, as a consequence, juvenile death (Hamilton *et al*. 1997). Embryonic development, however, is normal in these animals. In the vibrator mouse, PI-TPα mRNA as well as protein levels were found to be 80% reduced due to an insertion of a transposable element into intron 4 of the PI-TPα gene. The level of PI-TPβ, an isoform of PI-TPα, was not altered. The authors suggested that a defect in a PI-TPα-dependent PI-turnover pathway may be implicated in specific neurodegenerative diseases.

In another study, deletion of PI-TPα was established by ablation of the gene in mice (Alb *et al*. 2003). Similar to vibrator mice, PI-TPα appeared not to be required for prenatal development of the PI-TPα$^{-/-}$ mice. However, after birth PI-TPα$^{-/-}$ mice showed a lower body mass, hypoglycaemia and spinocerebellar degeneration characterized by reactive gliosis of the cerebellum and brain stem and white and grey matter deficits in the spinal cord. Reportedly, PI-TPα$^{-/-}$ mice do not survive beyond 14 days because of ongoing neurodegeneration. It can be concluded from these studies that, although expression of PI-TPα is not essential for normal embryonic brain development (probably due to *in utero* supply of maternal nutrients), this protein is necessary for normal development and maintenance of neural tissues in newborn mice.

Previously, it was shown that NIH3T3 fibroblasts overexpressing PI-TPα (SPIα cells) exhibited an increased survival upon induction of apoptosis *in vitro* (Snoek *et al*. 1999). Moreover, medium conditioned by SPIα cells protected wild-type cells from UV- and TNFα-induced apoptosis. It was shown that the overexpression of PI-TPα stimulated the

activation of a PI-specific PLA_2 leading to the release of arachidonic acid. As COX-2 inhibition reduced the production of the PI-TPα-dependent survival factors, it was concluded that arachidonic acid is the actual precursor of the survival factor (Schenning *et al*. 2004).

Although PI-TPα is ubiquitously expressed in mammalian tissues, it is to be noted that highest levels are found in brain (van Tiel *et al*. 2004). As demonstrated in the present study, PI-TPα is expressed in both cortex as well as spinal cord at levels significantly higher than in liver (Fig. 5). Moreover, we have shown by immunocytochemistry that PI-TPα can be detected in cultured primary motor neurons as well as in astrocytes (Fig. 6). On the basis of these observations it is tempting to hypothesize that a similar PI-TPα-driven production of a neuroprotective factor is operative in brain tissue. This hypothesis is supported by the following data.

First, besides PI-TPα, also the other two proteins critically involved in the PI-TPα-dependent generation of the survival factor(s) (i.e. COX-2 and PLA_2) are known to be expressed in brain under normal conditions (Yasojima *et al*. 1999; Sun *et al*. 2004). Moreover, astroglial and neuronal COX-2 was found to be up-regulated in Parkinson's and Alzheimer's diseased brains (Maihofner *et al*. 2003; Teismann *et al*. 2003; Hoozemans and O'Banion 2005), whereas PLA_2 and COX-2 mRNA and protein levels are markedly up-regulated in early stage amyotrophic lateral sclerosis (ALS) and ALS transgenic mice models (Yasojima *et al*. 2001; Maihofner *et al*. 2003; Kiaei *et al*. 2005). Not to deny the 'dual role' of COX-2 in the pathophysiology of neurodegenerative disease (Consilvio *et al*. 2004), at least in respect to the production of the PI-TPα-dependent survival factor, COX-2 expression is hypothesized to constitute a beneficial rather than a detrimental feature.

Second, there is accumulating evidence for the emerging role of the endocannabinoid system and endocannabinoid signalling in the maintenance of neuronal integrity and function (Pazos *et al*. 2005). From previous studies using mouse fibroblasts, evidence was obtained that (one of) the PI-TPα-dependent survival factor(s) is a COX-2-dependent endocannabinoid (Schenning *et al*. 2004). As reviewed by (van der Stelt *et al*. 2002), endocannabinoids are known to protect against acute brain damage most likely by acting on the cannabinoid (-like) receptors which are widely distributed in the CNS (Tsou *et al*. 1998). In addition, the neuroprotective effect of cannabinoids has also been reported in models of Huntington's disease, ALS, Parkinson's and Alzheimer's disease (Lastres-Becker *et al*. 2003; Raman *et al*. 2004; Lastres-Becker *et al*. 2005; Ramirez *et al*. 2005; Weydt *et al*. 2005). In our study, the broad-spectrum GPCR antagonist suramin partially inhibited the neuroprotective activity of CMα on motor neurons, indicating that GPCR activation may be similarly involved in the neuroprotective action of CMα on these cells. However, the identification of the specific GPCR involved, and the putative role of CB1R in

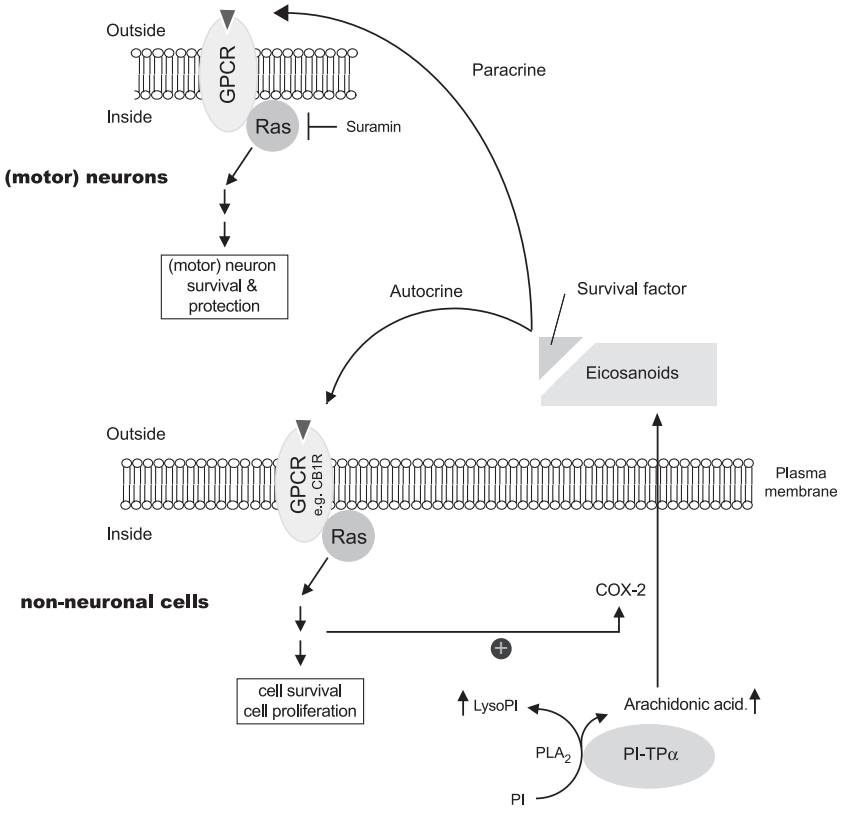

Fig. 7 Hypothetical regulatory role of phosphatidylinositol transfer protein α (PI-TPα) in the production of a neuroprotective eicosanoid in the CNS. High expression of PI-TPα results in activation of a phosphatidylinositol (PI)-specific phospholipase A_2 (PLA_2) and, as a consequence, increased production of arachidonic acid and lysophosphatidylinositol (LysoPI). Arachidonic acid is converted by cyclooxygenase-2 (COX-2) to yield eicosanoids including the survival factor. In turn, by stimulating G-protein coupled receptors (GPCRs), this factor is proposed to enhance cell survival and to confer neuroprotection in both an autocrine and a paracrine manner. In addition, the survival factor-mediated activation of GPCRs may result in an up-regulation in COX-2 expression, thus creating a positive feedback loop. CB1R, cannabinoid 1 receptor.

the neuroprotective effect of CMα, remains to be elucidated. Although the CB1R antagonist rimonabant strongly attenuated the CMα-induced neuroprotective effect, the antagonist itself resulted in a neurotoxic effect in control neurons, indicating that primary motor neurons depend on CB1R activation. This supports our hypothesis that the survival factors in CMα may display agonistic activity on CB1 (-like)Rs and thereby regulate neuronal protection and maintenance.

Finally, using mouse fibroblasts it was shown that the PI-TPα-dependent survival factor is able to act by autocrine as well as paracrine pathways (Schenning *et al*. 2004). Although it cannot be excluded that the neuroprotective factors are produced elsewhere in the body, we propose on the basis of our immunocytochemical and Western blot analysis, a paracrine protection pathway, in which astrocytes are likely candidates in providing the neuroprotective factor to neuronal cells. In this respect it should be emphasized that for optimal production and/or activation events, all proteins involved in the processes should be expressed at an appropriate level in particular cells.

Our data, together with the notion that all components required to produce the survival factor are present in brain, support our hypothesis that the PI-TPα-dependent survival factor may be implicated in the survival of (motor) neurons both in health and disease (see Fig. 7). We conclude that PI-TPα is a promising target to be evaluated in studies to the prevention and treatment of neurodegenerative disorders.

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