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The anti-apoptotic activity associated with phosphatidylinositol transfer protein α activates the MAPK and Akt/PKB pathway

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ABSTRACT

The conditioned medium (CM) from mouse NIH3T3 fibroblast cells overexpressing phosphatidylinositol transfer protein α (PI-TPα; SPIα cells) demonstrates an increased anti-apoptotic activity compared with CM from wild type NIH3T3 (wtNIH3T3) cells. As previously shown, the anti-apoptotic activity acts by activating a G protein-coupled receptor, most probably a cannabinoid 1 (CB1)-like receptor as the activity was blocked by both pertussis toxin and rimonabant [M. Schenning, C.M. van Tiel, D. Van Manen, J.C. Stam, B.M. Gadella, K.W. Wirtz and G.T. Snoek, Phosphatidylinositol transfer protein alpha regulates growth and apoptosis of NIH3T3 cells: involvement of a cannabinoid 1-like receptor, J. Lipid Res. 45 (2004) 1555–1564]. The CB1 receptor appears to be expressed in mouse fibroblast cells, at levels in the order SPIα>wtNIH3T3>SPIβ cells (i.e. wild type cells overexpressing PI-TPβ). Upon incubation of SPIβ cells with the PI-TPα-dependent anti-apoptotic factors, both the ERK/MAP kinase and the Akt/PKB pathway are activated in a CB1 receptor dependent manner as shown by Western blotting. In addition, activation of ERK2 was also shown by EYFP-ERK2 translocation to the nucleus, as visualized by confocal laser scanning microscopy. The subsequent activation of the anti-apoptotic transcription factor NF-κB is in line with the increased resistance towards UV-induced apoptosis. On the other hand, receptor activation by CM from SPIα cells was not linked to phospholipase C activation as the YFP-labelled C2-domain of protein kinase C was not translocated to the plasma membrane of SPIβ cells as visualized by confocal laser scanning microscopy.

1. Introduction

Phosphatidylinositol transfer protein α (PI-TPα) is a small, ubiquitously expressed protein belonging to a family of highly conserved proteins that in vitro transfers phosphatidylinositol (PI) and phosphatidylcholine (PC) between membranes [2,3]. In mammalian tissues, two soluble, highly homologous isoforms are identified: PI-TPα, localized in the nucleus and cytosol and PI-TPβ, associated with the Golgi membrane [4–7]. Cellular functions of these proteins comprise the stimulation of secretory vesicle formation from isolated Golgi membranes, PI metabolism and inositol lipid signalling [8–11]. Furthermore, a decreased expression of PI-TPα and PI-TPβ was demonstrated in aged brain and in Parkinson's disease, linking PI-TPs to neurodegenerative diseases [12–14]. This was supported by the finding that PI-TPα$^{-/-}$ mice died within 14 days after birth as a result of spinocerebellar disease characteristics, hypoglycemia, and intestinal steatosis [13]. Lethality of PI-TPβ gene ablation and difference in localization emphasizes that the PI-TP isoforms have separate physiological functions. A reduced expression of PI-TPα in rat WRK mammary tumour cells was reflected in a decreased rate of proliferation [9]. On the other hand, wtNIH3T3 mouse fibroblast cells expressing an increased level of PI-TPα (SPIα cells) have an enhanced rate of proliferation and are highly resistant towards UV- or tumour necrosis factor α-induced apoptosis [1,10]. We previously showed that the SPIα cells secrete mitogenic and anti-apoptotic arachidonic metabolites, the production of which is partially dependent on cyclooxygenase 2 [1]. The anti-apoptotic activity acts via activation of a G protein-coupled receptor, more specifically a cannabinoid 1-like receptor, as it was blocked by pertussis toxin and specific CB1 receptor antagonist rimonabant [1].

Several signalling pathways linking GPCRs (i.e. CB1 receptor) activation to cell survival have been described. It was shown that upon CB1 receptor activation the p42/p44 mitogen-activated protein kinase

Abbreviations: PI-TP, phosphatidylinositol transfer protein; PI, phosphatidylinositol; PC, phosphatidylcholine; PLA, phospholipase A; MAPK, mitogen-activated protein kinase; ERK, extracellular signalling related kinase; IAP, inhibitor of apoptosis protein; PKB, protein kinase B; PKA, protein kinase A; PKC, protein kinase C; YFP, yellow fluorescent protein; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle medium; NCS, newborn calf serum; DBB, DMEM containing 0.1% bovine serum albumin; CM, conditioned medium; PBS, phosphate buffered saline; PMSF, phenylmethanesulphonylfluoride; CB1, cannabinoid 1; GPCR, G protein-coupled receptor

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(MAPK) pathway plays an important role in the growth and survival of eukaryotic organisms [15–20]. The MAP kinase pathway is stimulated upon binding of extracellular signals to both tyrosine kinase receptors, G protein-coupled receptors and cytokine receptors. Receptor activation leads to the subsequent phosphorylation of Ras, Raf, MEK (MAPKK), and extracellular signalling related kinase (ERK1/2) (reviewed in [21]). Upon activation of MEK, the MEK/ERK complex dissociates by which ERK1/2 is phosphorylated and translocated into the nucleus where it phosphorylates transcription factors [22–24]. It has been reported that ERK affects apoptosis by promoting expression of IAPs (inhibitor of apoptosis protein) [25–27].

A second signalling pathway linking CB1 receptor activation to cell survival is the Akt/PKB pathway [15]. Similar to p42/p44 MAPK, Akt/PKB has emerged as a key regulatory factor in several cellular functions such as cell growth, transcriptional regulation and cell survival [28]. Akt/PKB is a downstream effector of PI 3-kinase, which is activated by tyrosine kinase and G protein-coupled receptors [29]. Upon receptor activation, PI 3-kinase gives rise to an increased PI (3,4,5)P_3 formation. PIP_3 recruits Akt/PKB to the plasma membrane where it is activated by phosphorylation. In addition, it has been reported that Akt/PKB can be activated via protein kinase A [30,31]. Upon activation, Akt/PKB has a direct effect on cell survival by inhibiting the pro-apoptotic Bcl-2 related protein, BAD and by inhibiting caspases 9. Furthermore, Akt/PKB affects apoptosis on a transcriptional level both by activation of NF-κB and CREB, which regulate the transcription of pro-survival genes like Bcl-xL, caspases inhibitors and by inhibition of YAK and Forkhead, which regulate the transcription of pro-apoptotic genes like JNK and Bax [32–36].

Here we provide evidence that the PI-TPα-dependent anti-apoptotic factors activate both the p42/p44 MAP kinase pathway and the Akt/PKB pathway via activation of the CB1 receptor. Subsequently, the anti-apoptotic transcriptional factor NF-κB is activated resulting in nuclear translocation.

2. Materials and methods

2.1. Materials

Anti-P-MAP kinase, anti-ERK1/2, anti-P-Akt/PKB, anti-IκBα and anti-P-IκBα antibodies were obtained from Cell Signaling technology. DMEM and Lipofectamine 2000 were obtained from Invitrogen. Cannabinoid 1 receptor antibody was a kind gift of Maurice R. Elphick (School of Biological Sciences, Queen Mary and Westfield College, University of London). YFP-ERK2 was a kind gift of Andrey Shaw (Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, USA). C2-YFP was a kind gift of Tobias Meyer (Department of Molecular Pharmacology, Stanford University Medical Center, Stanford, USA). The cannabinoid receptor antagonist SR141716A was a kind gift of Dr. G. van Zadelhoff (Section Bioorganic Chemistry, Bijvout Center, Utrecht Univerity).

2.2. Cell culture

All cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% newborn calf serum (NCS) and buffered with 44 mM $NaHCO_3$. Cells were maintained at 7.5% CO_2 at 37 °C in a humidified atmosphere. Wild type cells used were designated ATCC CRL 1658 NIH3T3 cells. wtNIH3T3 mouse fibroblast cells overexpressing PI-TPα (SPIα cells) were obtained as described in Snoek et al. [10]. The mouse fibroblast cells overexpressing PI-TPβ (SPIβ cells) were obtained as described in van Tiel et al. [37].

2.3. Preparation of conditioned medium

Cells were grown to 90% confluency in 150 cm^2 dishes. After washing the cells twice with PBS, the medium was replaced with 13 ml of *D*MEM/*b*icarbonate containing 0.1% *b*ovine serum albumin (DBB medium). After 24 h the medium was collected and centrifuged (10 min at 1000 rpm) to remove floating cells. The supernatant is the conditioned medium (CM). Under standard conditions 90% confluent cells were incubated with CM derived from an identical surface of cells (i.e. 9.5 cm^2 of cells per well of a 6-well dish was incubated with the amount of CM derived from 9.5 cm^2 of cells).

2.4. Induction of apoptosis by UV irradiation

Cells were seeded in 6-well plates and grown for 48 h until ca 90% confluency. Growth medium was removed and cells were washed with PBS. Prior to UV irradiation cells were incubated with either DBB medium or conditioned medium for 4 h. The medium was removed and the cells were given a dose of UV light (200 J/m^2) using a Stratalinker (Stratagene). After UV irradiation the cells were incubated with DBB at 37 °C. At the indicated time points cell death was morphologically determined as the percentage of cells that are in the process of blebbing. In addition, apoptosis was characterized by staining with 4′,6-diamidino-2-phenyindole (DAPI) visualizing condensed DNA or stained with annexin V visualizing phosphatidylserine externalization.

2.5. Membrane isolation

Cells were grown to 90% confluency in 150 cm^2 dishes. After washing the cells twice with PBS, the cells were scrapped off in 3 ml lysis buffer (0.1% NP40, 20 mM Tris-HCl, 150 mM NaCl, pH 7.6). The cell suspension was sonicated (two 10-s bursts) on ice. Nuclei and cell debris were removed from the homogenate by centrifugation at 17,500 ×g from 10 min. The resulting supernatant was centrifuged at 100,000 ×g for 3 h at 4 °C (SW40 rotor, Beckman ultracentrifuge). The membrane pellet was solubilized in buffer (1% Triton X-100, 20 mM Tris-HCl, pH 7.6) by sonication (three 10-s bursts on ice) and the protein content was determined using the Bradford assay. Equal amounts of membrane proteins were subjected to non-reducing SDS-PAGE on a 12% gel and Western blot analysis was performed using a CB1 receptor specific antibody. The blot was stained with ponceau S and scanned to determine whether equal amounts of protein were analysed. The specificity of the CB1 receptor staining was established by pre-absorption of the affinity-purified antibody with the CB1 C-terminal peptide antigen (20 μM) for 1 h before incubation.

2.6. Sample preparation for Western blot analysis

Cells were grown in 21 cm^2 dishes to 80–90% confluency, serum starved for 4 h and incubated with CM for the indicated times. Cells were washed twice with PBS (ice-cold) and lysed in 20 mM Tris–HCl pH 7.5 containing 0.1% (v/v) NP_{40}, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 50 mM NaF, 1 mM aprotinin and 1 mM PMSF. Cells were scraped off and transferred to an Eppendorf tube. Cell lysates were centrifuged at 17,500 ×*g* for 10 min at 4 °C and the protein content of the supernatant fractions determined using the Bradford assay [38]. Equal amounts of supernatant proteins (50 μg) were subjected to SDS-PAGE on a 12% gel and Western blot analysis was performed using specific antibodies. To determine whether equal amounts of protein were analysed, blot were stained with ponceau S and scanned. Bands on the immunoblot were quantified using a Bio-Rad GS700 imaging densitometer equipped with an integrating program.

2.7. Fluorescence microscopy

Cells were seeded in 6-well plates containing 24 mm Ø coverslips. The next day, cells were transfected with 1 μg purified plasmid DNA using Lipofectamine 2000 (Invitrogen, Breda, The Netherlands). One day after transfection the coverslip was mounted in a home made chamber or an Attofluor cell chamber from Molecular Probes (Leiden, The Netherlands). Cells were observed and imaged on a LSM510 (Zeiss, Germany) confocal laser scanning microscope. A Zeiss 63′ oil-immersion objective (Plan-Apochromat, NA 1.4) was used. YFP was excited using the 488 nm laser line, which was reflected onto the sample by a 488 nm dichroic mirror. YFP fluorescence was passed through a 505–550 nm bandpass filter and detected with a pinhole setting corresponding to 1 airy unit.

2.8. Data analysis

The average nuclear fluorescence (YFP-ERK2) or membrane/cytosolic fluorescence (C2-YFP) of a single cell was measured at every time-point by selecting a region of interest (ROI) in the right area using the Zeiss LSM510 software version 3.2. The initial value was normalized to 1. Graphs were prepared using Kaleidagraph 3.6 (Synergy software, Reading, PA).

3. Results

3.1. Anti-apoptotic activity of CM from SPIα and wtNIH3T3 cells

Previously, we have shown that wtNIH3T3 cells overexpressing PI-TPα (SPIα cells) produce and secrete potent mitogenic and anti-apoptotic factors [1]. The mechanism by which PI-TPα regulates the production of these mitogenic and anti-apoptotic factors is discussed in Schenning et al. [1]. As shown in Fig. 1, SPIβ cells are fully protected against UV-induced apoptosis upon incubate for 4 h with CM from SPIα cells. Since wtNIH3T3 cells contain an endogenous level of PI-TPα and are known to produce mitogenic ant anti-apoptotic factors [39,40], CM from wtNIH3T3 cells also expresses anti-apoptotic activity, yet to a lesser extent (Fig. 1). This difference in anti-apoptotic activity reflects the levels of PI-TPα in these cells.

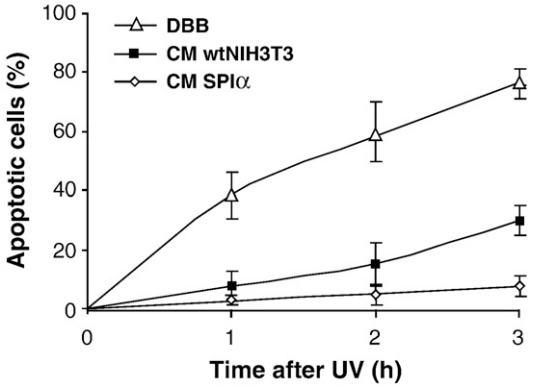

Fig. 1. Survival of SPIβ cells upon induction of apoptosis by ultraviolet (UV) irradiation. SPIβ cells were grown to 90% confluency and incubated from 4 h at 37 °C with DMEM/Bic/0.1% bovine serum albumin (DBB), CM from wtNIH3T3 or CM from SPIα cells. After removing the media, the cells were irradiated with a dose of UV light (200 J/m^2), fresh DBB was added to the cells and incubated for 3 h at 37 °C. The number of apoptotic cells (blebbing) was determined by visual analysis at the indicated times. Results±SD represent the mean values of at least three experiments.

3.2. CB1 receptor in wtNIH3T3, SPIα and SPIβ cells

Previously it was shown that the CB1 receptor antagonist SR141716A inhibits the anti-apoptotic activity of CM from SPIα cells [1]. Western blot analysis of the membrane fractions of wtNIH3T3, SPIα and SPIβ cells using affinity-purified antibodies against the CB1 receptor revealed immunoreactive bands at ~41, ~53 and ~62 kDa. The antibody used has been raised against the C-terminal 13 amino acids of the receptor and has previously been shown to be specific for the CB1 receptor [41,42]. The immunoreactive ~53 kDa band corresponds with the predicted molecular weight of the full length CB1 receptor, whereas the ~62 kDa band is consistent with the glycosylated isoform of the CB1 receptor detected previously in rat brain [41–43] (Fig. 2). The immunoreactive ~41 kDa band may have resulted from previously described N-terminal enzymatic degradation or may be an alternatively spliced isoform of the CB1 receptor comparable to the CB1A isoform in humans [44,45] (Elphick M.R., personal communication). The specificity of CB1 staining was established by blocking the affinity-purified antibody with the CB1 C-terminal peptide antigen. This pre-absorption abolished CB1 staining (all three immunoreactive bands) when using the antibody for Western blot analysis. The full length CB1 receptor appears to be prominently present in SPIα cells and to some extent in wtNIH3T3 cells. Moreover, the immunoreactive band at ~41 kDa and ~62 kDa appear to be present to equal extents in wtNIH3T3 and SPIα cell and to a lesser extent in SPIβ cells.

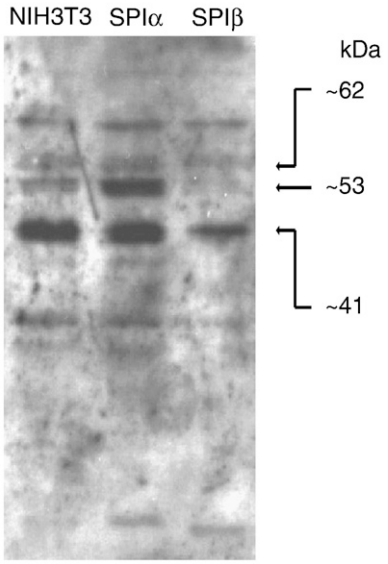

Fig. 2. Expression of cannabinoid 1 receptor in wtNIH3T3, SPIα and SPIβ cells. Cells were grown to 90% confluency and membrane enriched fractions were prepared as described in Materials and methods. Equal amounts of membrane proteins (40 μg) from wtNIH3T3, SPIβ and SPIβS262A cells were separated on an 8% polyacrylamide, Tris–glycine gel under non-reducing conditions. The proteins were transferred from the gel to nitrocellulose and subjected to Western blot analysis using an affinity-purified cannabinoid 1 receptor antibody. To check whether equal amounts of protein were analysed, blots were stained with ponceau S and scanned.

3.3. P42/P44 MAP kinase activation

Given that both CM from wtNIH3T3 and SPIα cells exhibited anti-apoptotic activity and since it has been reported that the CB1 receptor activates the p42/p44 MAP kinase pathway [46], we investigated the involvement of this pathway. Incubation of SPIβ cells with CM from SPIα cells gave rise to a rapid phosphorylation of p42/p44 MAP kinase over a time period of 30 min (Fig. 3A; left panel). After 1 h the level of

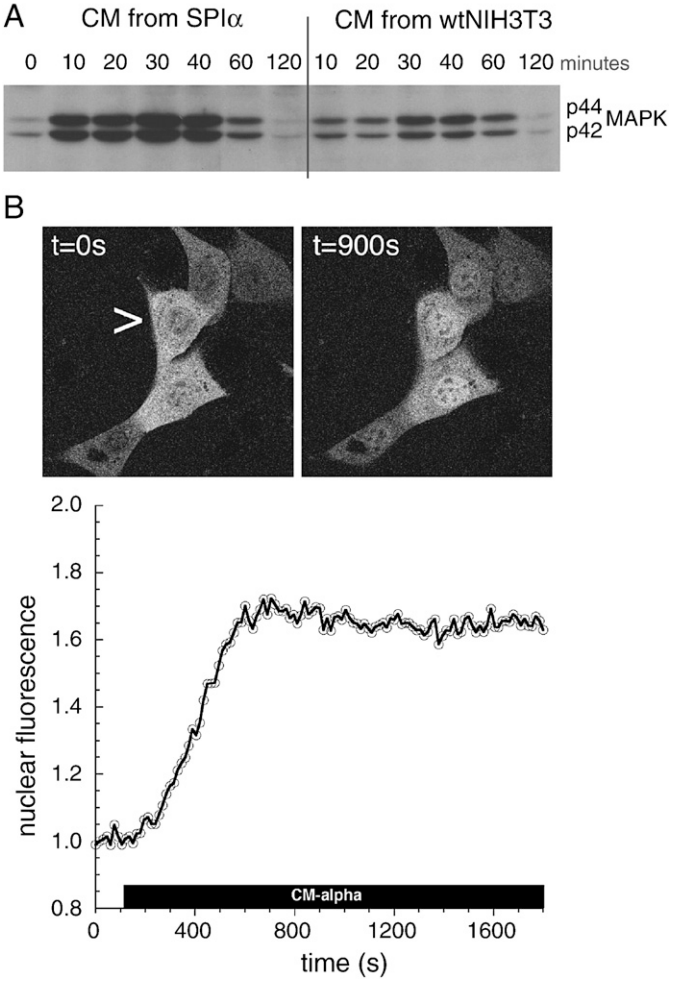

Fig. 3. A. Phosphorylation of p42/p44 MAP kinase in SPIβ cells upon incubation with CM from wtNIH3T3 or SPIα cells. SPIβ cells were grown to 90% confluency, serum starved for 4 h and incubated for the indicated times with CM from SPIα or wtNIH3T3 cells at 37 °C. Equal amounts of cell lysate protein (25 μg) were subjected to SDS-PAGE followed by Western blot analysis using a p42/p44 MAP kinase specific antibody. To check whether equal amounts of protein were analysed, blots were stained with ponceau S and scanned. Representative experiment performed in triplicate. B. Confocal images of SPIβ cells transfected with EYFP-ERK2 before (t=0 s) and after stimulation with CM from SPIα cells (t=900 s). The relative fluorescence intensity in the nucleus of the cell indicated with the arrowhead is quantified and shown in the graph (initial fluorescence is normalized to 1). The black bar indicates the presence of CM from SPIα cells. Width of a single image is 146 μm. Representative experiment performed in triplicate.

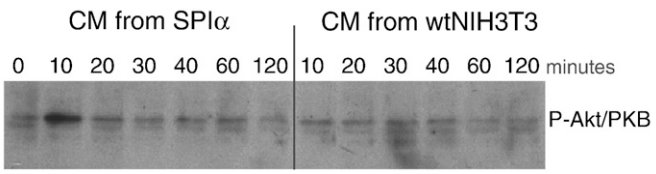

Fig. 4. Phosphorylation of Akt/PKB in SPIβ cells upon incubation with CM from wtNIH3T3 or SPIα cells. SPIβ cells were grown to 90% confluency, serum starved for 4 h and incubated for the indicated times with CM from SPIα or wtNIH3T3 cells at 37 °C. Equal amounts of cell lysate protein (50 μg) were subjected to SDS-PAGE followed by Western blot analysis using a P-Akt/PKB specific antibody. To check whether equal amounts of protein were analysed, blots were stained with ponceau S and scanned. Representative experiment performed in triplicate.

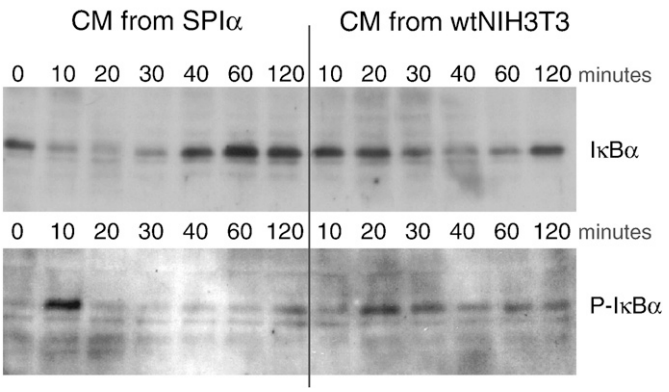

Fig. 5. NF-κB activation in SPIβ cells upon incubation with CM from wtNIH3T3 or SPIα cells. SPIβ cells were grown to 90% confluency, serum starved for 4 h and incubated for the indicated times with CM from SPIα or wtNIH3T3 cells at 37 °C. Equal amounts of cytosolic protein (25 μg) were subjected to SDS-PAGE followed by Western blot analysis using an IκBα or P-IκBα specific antibody visualizing clearance from the cytosol. To check whether equal amounts of protein were analysed, blots were stained with ponceau S and scanned. Representative experiment performed in triplicate.

phosphorylation returned to normal. For comparison, phosphorylation was much less upon incubation with CM from wtNIH3T3 cells (right panel) most likely reflecting the difference in anti-apoptotic activity.

Previously, it was shown that activation of ERK2 (p42 MAP kinase) is paralleled by translocation of a fluorescent-tagged ERK2 into the nucleus [47]. To visualize the activation of the p42/p44 MAP kinase pathway with high spatial–temporal resolution in single living cells, SPIβ cells were transiently transfected with YFP-tagged ERK2 [48]. In resting cells, ERK2 is predominantly located in the cytoplasm. Upon addition of CM from SPIα cells, a clear accumulation of ERK2 in the nucleus was observed (Fig. 3B). A rapid initiation of the ERK2 translocation was shown, attaining a maximum within 10 min (Fig. 3B). These data agree very well with the rapid activation of the p42/p44 MAP kinase pathway observed by Western blot analysis. Combined, these data show that the additional anti-apoptotic factors present in CM from SPIα cells induce a transient and increased activation of the p42/p44 MAP kinase pathway compared with CM from wtNIH3T3 cells.

3.4. Akt/PKB and NF-κB activation

To further establish the mode of action of the PI-TPα-dependent anti-apoptotic activity, the involvement of the Akt/PKB signalling pathway was studied by Western blot analysis using the antibody against Ser-473. Upon incubation of SPIβ cells with CM from SPIα cells, Akt/PKB was phosphorylated to a maximum extent at 10 min (Fig. 4). For comparison, CM from wtNIH3T3 cells induced very limited Akt/PKB phosphorylation.

Since the activation of both the p42/p44 MAP kinase pathway and the Akt/PKB pathway can result in NF-κB activation, we investigated the effect of CM from wtNIH3T3 and SPIα cells on the activation of this anti-apoptotic transcription factor. NF-κB is retained in the cytoplasm in an inactive form by association with IκBα, the intracellular NF-κB inhibitor. Upon phosphorylation of IκBα by IKK (IκB kinase complexes), the IκBα/NF-κB complex dissociates and p-IκBα is ubiquitinated and subsequently degraded in proteasomes. Phosphorylation of IκBα at Ser32 and the clearance of IκBα from the cytosolic fraction indicate NF-κB activation. In agreement with the activation of the above pathways, incubation with CM from SPIα cells (10 min) gave rise to phosphorylation of IκBα (Fig. 5; lower left panel). Concomitantly, IκBα was cleared from the cytosol and reappeared again after 40 min (Fig. 5; upper left panel). For comparison, incubation of SPIβ cells with CM from wtNIH3T3 cells showed less and a slower phosphorylation of IκBα and some clearance of IκBα from the cytosol (Fig. 5; right panels). This indicates that the supplementary anti-apoptotic factors present in CM from SPIα cells result in an increased NF-κB activation.

To further establish the role of the CB1 receptor, we investigated the effect of SR141716A (a specific CB1 receptor antagonist) on the activation of the p42/p44 MAP kinase, Akt/PKB, and NF-κB pathways by CM from SPIα cells. As previously shown, incubation of SPIβ cells with CM from SPIα cells activated all three pathways within 10 min (Fig. 6; left panel). Upon incubation of SPIβ cells with CM from SPIα cells in the presence of SR141716A, the activation of both the p42/p44 MAP kinase pathway and the Akt/PKB pathway was significantly reduced (Fig. 6; right panel). Subsequently, NF-κB inhibitor protein IκBα was phosphorylated to a lesser extent. This implies that the CB1 receptor mediates the effect of the PI-TPα-dependent anti-apoptotic factors on the p42/p44 MAP kinase, Akt/PKB, and NF-κB pathways.

3.5. Phospholipase C activation

Since several reports show that PI-TPα is involved in G protein-coupled receptor-mediated activation of phospholipase C (PLC) [11], we investigated whether the PI-TPα-dependent survival factor could play a role in the activation of PLC. It has been demonstrated that the calcium-sensitive C2 domain of protein kinase C fused to YFP (C2-YFP) is a sensitive reporter of PLC-mediated release of intracellular calcium

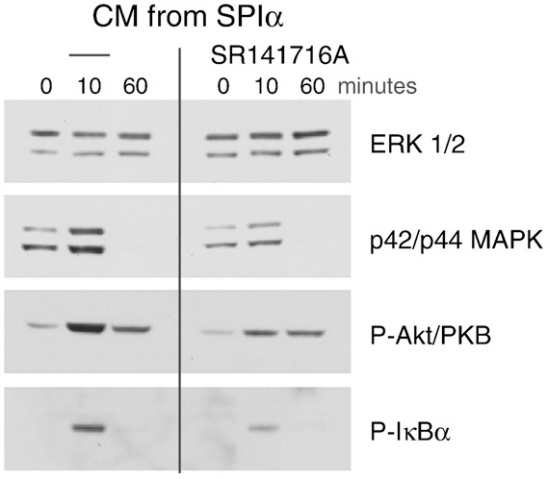

Fig. 6. Effect of CB1 receptor antagonist SR141716A on the activation of the p42/p44 MAP kinase, Akt/PKB, and NF-κB pathways upon incubation with CM from SPIα cells. SPIβ cells were grown to 90% confluency and incubated for the indicated times with CM from SPIα cells in the presence or absence of SR141716A. Equal amounts of cell lysate protein were subjected to SDS-PAGE followed by Western blot analysis using an ERK1/2, p42/p44 MAP kinase, P-Akt/PKB, or P-IκBα specific antibody visualizing pathway activation. To check whether equal amounts of protein were analysed, blots were stained with ponceau S and scanned. Representative experiment performed in triplicate.

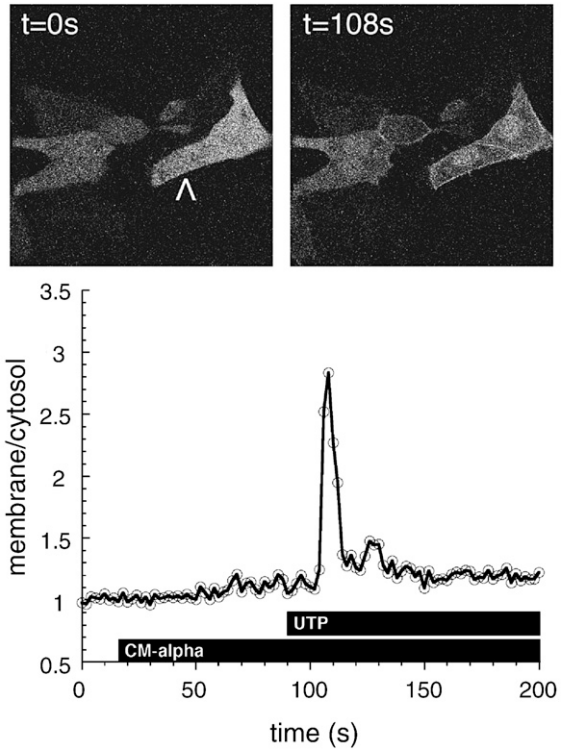

Fig. 7. Confocal images of SPIβ cells transfected with the C2 of protein kinase C tagged with YFP before (t=0s) and just after stimulation with 100 μM UTP (t=108s). The fluorescence ratio of membrane/cytosol of the cell indicated with the arrowhead is quantified and shown in the graph (initial fluorescence is normalized to 1). The black bars indicate the presence of CM from SPIα and UTP in the medium. Width of a single image is 146 μm.

[49,50]. Upon an increase in the intracellular calcium concentration, the C2-YFP is translocated to the plasma membrane. SPIβ cells transiently transfected with C2-YFP displayed a homogeneous distribution of yellow fluorescence in the cytoplasm and nucleus (Fig. 7A). Upon incubation with CM from SPIα cells the distribution remained unchanged. However, upon addition of uridine triphosphate (UTP; a potent PLC activator) the C2-YFP accumulated at the plasma membrane. This indicates an increase in intracellular calcium followed by PLC activation (Fig. 7A). Quantification of the ratio of membrane/cytosol fluorescence showed no change in the ratio due to CM from SPIα but a fast and transient increase in the ratio upon addition of UTP (Fig. 7B). These data indicate that CM from SPIα does not activate PLC. This is in agreement with previous observations that the activation of the CB1 receptor is not linked to PLC activation [51].

4. Discussion

We have previously shown that wtNIH3T3 mouse fibroblast cells overexpressing PI-TPα (SPIα cells) produce (a) COX-2-dependent eicosanoid(s), which inhibit(s) UV-induced apoptosis of SPIβ cells via activation of a G protein-coupled receptor (GPCR). Here we show that in line with SPIα cells expressing 2–3 fold higher levels of PI-TPα compared with wtNIH3T3 cells, CM from SPIα cells displays a more pronounced effect on the survival of SPIβ cells than CM from wtNIH3T3 cells (Fig. 1). The observation that an increase in the concentration of CM from wtNIH3T3 cells did not result in an anti-apoptotic effect comparable to CM from SPIα cells suggests that CM from SPIα cells contains additional anti-apoptotic factors which may be absent from CM from wtNIH3T3 cells [unpublished data] [52]. This is confirmed by TLC analysis of neutral lipid extracts of CM from wtNIH3T3 and SPIα cells [1]. As a control, the level of PI-TPα was reduced in wtNIH3T3 cells by RNAi (data not shown). Unfortunately, the reduced level of PI-TPα significantly diminished the growth rate of the wtNIH3T3 cells and led to a high level of cell death. Therefore we were unable to evaluate the anti-apoptotic activity of the medium conditioned by cells with a reduced level of PI-TPα. On the other hand, these results agree with the observation that WRK-1 rat mammary tumour cells, which as a result of transfection expressed approximately 25% less PI-TPα than control clones, showed a decreased growth rate [9].

The inhibitory effect of the antagonist SR141716A indicates that the GPCR activated by the anti-apoptotic factors present in CM from SPIα cells is the cannabinoid 1 (CB1) or a cannabinoid 1-like receptor [1]. Here we report that wtNIH3T3 mouse fibroblast cells, SPIα and SPIβ cells express the CB1 receptor although to different extents. Highest full length CB1 receptor levels were detected in SPIα cells, intermediate levels in the wild type cells, whereas SPIβ cells exhibit no full length CB1 receptor. The immunoreactive band detected at ~41 kDa (Fig. 2) may be due to either alternative splicing of the mouse CB1 receptor gene or N-terminally truncation of the receptor. Evidence for an alternative splicing of the CB1 receptor gene has previously been reported in human cells [53]. On the other hand, several studies report on the amino-terminal processing of the CB1 receptor [45,54]. Due to the length of the N-terminal segment, the CB1 receptor cannot be efficiently translocated across the ER membrane. This leads to the degradation of the CB1 receptor by proteasomes and hence, to a low expression level at the plasma membrane. In accordance, it was shown in baby hamster kidney cells that a large number of the CB1 receptors are N-terminally truncated prior to ER translocation [54]. Shortening of the N-terminal tail, leaving a ~41 kDa CB1 receptor, greatly enhances the stability and cell surface expression of the receptor without affecting receptor binding to cannabinoid ligands [45]. In line with this, the effect of the CB1 receptor antagonist SR141716A on the protective effect of CM from SPIα cells indicates that SPIβ cells have a functional CB1 receptor. Since no full length CB1 receptor is detected in SPIβ cells it may well be that the cross-reactive ~41 kDa protein detected in mouse fibroblast cells is a CB1 receptor which is N-terminally truncated. Furthermore, the observed change in the membrane–lipid composition of SPIβ cells (i.e. a shift from short chain to long chain ceramide/SM species [55] may hamper translocation of the N-tail of the CB1 receptor across the ER membrane resulting in rapid degradation of this full length receptor and a reduced membrane expression in SPIβ cells. In line with the low level of CB1 receptor in SPIβ cells and its high sensitivity towards UV-induced apoptosis, several studies report that reduced levels or inhibition of this receptor enhances apoptosis [52,56,57].

Cannabinoids exert most of their effects by binding the CB1 receptor at the plasma membrane thereby inhibiting adenylate cyclase (AC) and N- and P/Q-type voltage-sensitive calcium channels (VSCC), as well as activating mitogen- and stress-activated protein kinase (ERK, JNK, p38) and Akt/PKB pathways [16–18,28,58,59]. In line with the fact that CM from SPIα cells is more effective in protecting SPIβ cells against apoptosis compared with CM from wtNIH3T3 cells (Fig. 1), Western blot analysis showed that CM from SPIα cells is much more effective in the activation of the anti-apoptosis p42/p44 MAP kinase and Akt/PKB pathways. In addition, CM from SPIα cells triggered a faster and more significant activation of the transcription factor NF-κB. The observation that SR141716A, a specific CB1 receptor antagonist, shows an inhibitory effect on the activation of these pathways by CM from SPIα cells implies that the effect is mediated by the CB1 receptor (Fig. 6). The activation of NF-κB is in agreement with the up regulation of cyclooxygenase-2 in SPIα cells [1] as the expression of this protein is controlled by NF-κB [60]. The activation appeared to be rather specific as phosphorylation of p38 MAP kinase was not observed (data not shown). NF-κB activation up regulates the transcription of pro-survival genes encoding c-IAP1, c-IAP2, and IXAP, the TNF receptor-associated factors (TRAF1 and TRAF2) and members of the Bcl-2 family, in addition to up regulating a gene promoting cell proliferation, cyclin D1 a positive regulator of G1-to-S-phase progression [61–63]. In line with this, the anti-apoptotic activity present in CM from SPIα cells is likely

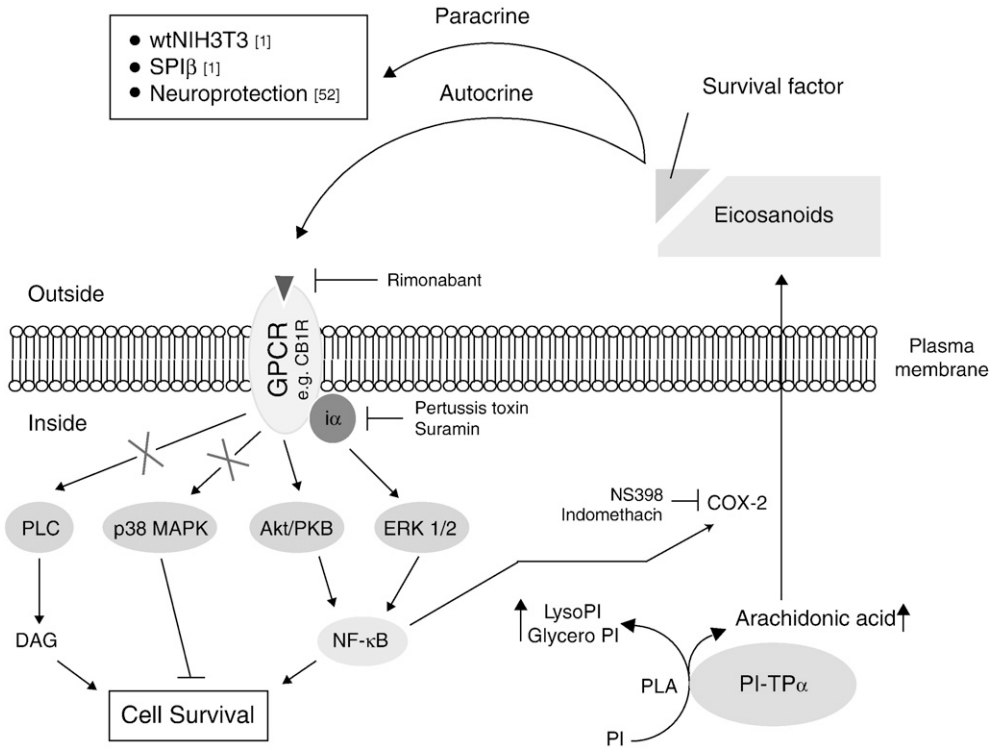

Fig. 8. The regulatory role of phosphatidylinositol transfer protein (PI-TPα) in the production of a bioactive eicosanoid. In the model presented, PI-TPα activates a PI-specific phospholipase A (PLA) leading to an increased release of arachidonic acid, which is subsequently converted into eicosanoids by COX-2 [1]. A part of these eicosanoids constitute the survival factor(s), which has anti-apoptotic activity by acting through the activation of a G protein-coupled receptor, possibly a CB1-like receptor. Upon receptor activation, the p42/p44 MAP kinase and Akt/PKB pathway are activated. On the other hand, the P38 MAP kinase and PLC pathways are not activated.

to exert its protective effect by the expression of all these proteins. Since the activation of the Akt/PKB pathway also affects transcriptional factors like CREB, YAK and Forkhead, the activation or inhibition of other pathways affecting cells survival and cell proliferation is likely.

Several studies show a relationship between PI-TPα and the G protein-couple receptor-mediated activation of phospholipase C (PLC) upon addition of PI-TPα to cytosol depleted cells [11,64]. By using SPIβ cells transfected with the plasmid containing cDNA encoding YFP-tagged C2 domain from PKC, we demonstrated that addition of CM from SPIα cells had no effect on the translocation of the fluorescent probe. Since the positive regulator of PLC (UTP) did result in translocation (Fig. 7), it appears that the cellular effect of the anti-apoptotic factor is independent of PLC activation. This is in line with the fact that CB1 receptor signalling leads to the activation of the G protein $G_i\alpha$ which does not activate PLCβ [65–68]. This observation does not necessarily contradict the studies by Cockcroft et al. that PI-TPα acts directly in the cell through activation of PLC [69]. In the latter case, it is proposed that PI-TPα is essential for enhanced formation of PI(4)P and PI(4,5)P_2 as substrates of PLC. The data we presented here is summarized in a model representing a possible mechanism by which cell survival depends on PI-TPα (Fig. 8). In the model presented, PI-TPα activates a PI-specific phospholipase A (PLA) [10]. This implies that, in addition to lysoPI and glyceroPI, a significant amount of arachidonic acid is produced because PI is highly enriched in this fatty acid [70]. Arachidonic acid is the main precursor for the synthesis of eicosanoids and is subsequently converted by COX-2 [1]. A part of these eicosanoids constitute the survival factor(s), which has/have anti-apoptotic activity by acting through the activation of a G protein-coupled receptor, most likely the CB1 or a CB1-like receptor. Upon receptor activation, the anti-apoptotic p42/p44 MAP kinase and Akt/PKB pathways are activated, whereas the PLC/DAG and p38 MAP kinase pathways remain unchanged. The subsequent activation of the transcription factor NF-κB, a positive regulator of COX-2, is in line with the increased levels of this enzyme observed in SPIα cells [1].

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