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Cyclic AMP-dependent down regulation of ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) in rat C6 glioma

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ABSTRACT

In this communication, we demonstrate that an increase in intracellular cAMP by 1) addition of dibutyrylic cAMP (dbcAMP), a membrane-permeable cAMP-analogue, or 2) activation of the β-adrenoceptor with (−)-isoproterenol, down regulates the levels of ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) mRNA, NPP1 protein and ecto-NPPase activity in rat C6 glioma cells. DbcAMP and (−)-isoproterenol inhibit NPP1 expression in a time and dose-dependent manner. After 48 h of stimulation, 1 mM dbcAMP or 5 μM (−)-isoproterenol decreases the amount of NPP1 protein by 75 ± 3% and 81 ± 1% respectively. Contrary to down regulation of NPP1, we observe an up regulation of glial fibrillary acidic protein (GFAP), a differentiation marker for astrocytic cells. Using specific inhibitors and activators, we have shown that Ca^{2+}, PKA, PI 3-K/PKB/GSK-3, Epac/Rap1/PP2A and MAP kinase modules are not involved in the inhibition of NPP1 gene expression.
The transcription factor *c-jun* is significantly reduced while *c-fos* becomes up regulated after cAMP elevation. However an electrophoretic mobility shift assay with the activator protein-1 motif present in the promoter of the rat NPP1 gene indicates that this motif is not involved in the cAMP-dependent inhibition of NPP1 expression. In conclusion, these results indicate that intracellular cAMP levels regulate the expression of NPP1 in rat C6 glioma cells by a signalling pathway that is different from the GFAP signal transduction pathway.

1. Introduction

Extracellular nucleotides are important signalling molecules regulating short-term and long-term cellular functions in the central nervous system (Zimmermann, 2006; Neary and Zimmermann, 2009). Nucleotide-dependent signalling is modulated by hydrolysis of extracellular nucleotides by cell surface-bound enzymes, such as ecto-ATPase (EC 3.6.1.3), ecto-ATPDase (EC 3.6.1.5), ecto-nucleotidase (EC 3.1.3.5) and ecto-nucleotide pyrophosphatase (EC 3.6.1.9)/phosphodiesterase (EC 3.1.4.1) (E-NPP) (Zimmermann, 2000; Goding et al., 2003; Yegutkin, 2008).

The E-NPP family contains seven structurally related ecto-enzymes, including NPP1, originally described by Takahashi et al. (1970) as the plasma cell differentiation antigen PC-1. It releases nucleoside 5′-monophosphate from a variety of nucleotides and their derivates (Goding et al., 2003; Yegutkin, 2008). NPP1 and NPP3 are type II membrane-bound ecto-enzymes with a short amino-terminal intracellular domain. NPP4–7 are type I transmembrane proteins with a short intracellular carboxyl-terminal domain. The extracellular domain only contains a phosphodiesterase activity (Bollen et al., 2000; Goding et al., 2003; Stefan et al., 2005). NPP2 is not a transmembrane protein but is secreted after removal of the N-terminal signal peptide (Jansen et al., 2005). Soluble forms have also been identified for NPP1, NPP3, NPP6 and NPP7, but the mechanisms for their generation remain to be elucidated (Belli et al., 1993; Yegutkin et al., 2003). Many tissues contain several NPP subtypes that are active in physiological and biochemical processes such as bone mineralization, nucleotide recycling, cell motility, angiogenesis and invasion (Goding et al., 2003).

The rat C6 glioma is a rapidly proliferating cell line with astrocytic, neuronal and oligodendrocytic progenitor properties. It is an experimental model system for astrocytes and glioblastoma multiforme (Grobben et al., 2002; Goding et al., 2003). Cyclic AMP-dependent induction of differentiation towards a type II astrocyte by membrane-permeable cAMP analogues or β-adrenoceptor agonists (Messens and Slegers, 1992; Roymans et al., 2000), occurs with PKA-independent and PI 3-K dependent GFAP expression, which is itself a marker for astrocytic differentiation (Anciaux et al., 1997; Roymans et al., 2001). Also, our laboratory has shown the presence of only NPP1 on the plasma membrane of C6 cells (Grobben et al., 1999).

To date, little is known about the pathways regulating NPP expression. PKA activity is directly affected by an increase in cAMP as a result of the activation of adenylate cyclase by G-protein coupled receptors. In addition, cAMP also activates Epac, an exchange protein directly activated by cAMP, resulting in activation of Rap1 (de Rooij et al., 1998; Mei et al., 2002). Another target is MAP kinase, which can be

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activated or inhibited by cAMP in a cell specific manner (Dugan et al., 1999).

Ultimately several cAMP-dependent or independent signalling pathways inhibit or activate transcription factors of the activator protein-1 (AP-1) family (Lamph et al., 1990; Moody et al., 1997). Promoter analysis found a putative AP-1 binding site in the rat NPP1 promoter and studies showed that treatment with the β-adrenoceptor agonist (-)-isoproterenol resulted in a decrease of *c-jun* mRNA level in C6 cells (Lee et al., 1999, 2001), whereas *c-fos* mRNA increases after cAMP elevation (Moody et al., 1997; Zhang et al., 2000).

In this communication we attempt to elucidate the signal transduction pathways that are involved in the cAMP-dependent down regulation of NPP1 expression in C6 cells and show that it differs from the pathway that induces GFAP.

2. Materials and methods

2.1. Materials

Nucleotides, nucleotide derivatives, (−)-isoproterenol and the PI 3-K inhibitor 2-(4-morpholinyl)-8-phenyl-4h-1-benzopyran-4-one (LY294002) were from Sigma Chemical Co. (USA). The inhibitors okadaic acid, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H89), 4-(4-fluorophenyl-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1h-imidazole (SB203580), 1,9-pyrazolanthrone (SP600125, JNK inhibitor II), 2′-amino-3′-methoxyflavone (PD98059) and 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra (acetoxymethyl)ester (BAPTA-AM) were from Calbiochem (Merck N.V., Belgium). 8-(4-chlorophenyl thio)-2′-O-methyl-cAMP (8-pCPT-2′-O-Me-cAMP) was from Biolog Life Science Institute (Bremen, Germany). [γ-^{32}P] ATP was obtained from Amersham (Amersham Pharmacia Biotech, UK). The polyclonal rabbit anti-GFAP antibody was from Dakopatts (Glostrup, Denmark) and the mouse monoclonal anti-GAPDH antibody from Bio Trend (Köln, Germany). Polyclonal anti-NPP1 antibody was kindly provided by Dr. H. Sakoda (Sakoda et al., 1999). Nuclear Extract Kit and TransAM AP-1 family kit were from Active Motif (Rixensart, Belgium). The BCA protein assay was from Pierce (Aalst, Belgium).

2.2. Cell culture

Rat C6 cells (ATCC, no. CCL 107) were obtained from ATCC (Manassas, USA) (passage no.: 40–49) and maintained in monolayer culture as described previously (Slegers and Joniau, 1996). Experiments were performed on C6 cells cultured in 96-well plates at 37 °C and 5% CO_2 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 (Invitrogen, UK), and 30 nM sodium selenite (Sigma Chemical Co., USA). Culture was in a humidified atmosphere of 5% CO_2 and at 37 °C. Cell numbers were measured in a haemocytometer after cell detachment with trypsin/EDTA in phosphate-buffered saline (PBS).

2.3. Immunoblotting

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. After stimulation, as described in the figure legends, and incubation for 48 h, the cultivation medium was removed and the cells were dissolved in SDS-PAGE sample buffer (2×) [40 mM Tris(hydroxylmethyl) aminomethane hydrochloride (Tris–HCl) (pH 6.8), 1% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 1 mM $NaVO_3$, 1 mM NaF, and 30 mM DTT]. Samples were boiled for 5 min and aliquots of cells analyzed by SDS-PAGE on a 10% (w/v) polyacrylamide gel. Proteins were electroblotted overnight onto a nitrocellulose membrane (Hybond-C pure, Amersham Bioscience, USA) and NPP1 detected with a polyclonal rabbit anti-NPP1 antibody (1:4000) as described previously (Sakoda et al., 1999). Induction of differentiation of C6 cells was evaluated by measurement of GFAP using a rabbit polyclonal antibody (1:20 000) against GFAP (DakoCytomation, Denmark). Following incubation of the membrane with primary antibodies against NPP1, GFAP and GAPDH, the blot was incubated with horseradish peroxidase-labeled anti-rabbit IgG, as a secondary antibody, and visualized by enhanced chemiluminescence (LumiGlo®, Cell Signalling Technology, UK) according to the manufacturer's instructions. Luminograms were recorded with the Gel Doc 2000 system (Bio-Rad, Hercules, USA) and analyzed with Image Quant (Molecular Dynamics, Amersham Biosciences, USA).

2.4. Semiquantitative RT-PCR

C6 cells were cultivated as described above and total RNA was isolated with RNeasy protect midi kit (Qiagen®, QIAGEN Benelux B.V., The Netherlands) according to the manufacturer's instructions. RNA was used as template in a one-step hot start RT-PCR (Qiagen®, QIAGEN Benelux B.V., The Netherlands). PCR was performed under conditions of linear kinetics. Primers were designed from the cDNA sequences (GenBank®: rat Wistar NPP1, AF320054; rat GAPDH, M17701) and synthesized at Eurogentec (Belgium). The sequences of the primers were as follows:

NPP1,
5′-ACCTCGGCTGCACATGTGAC-3′ (sense primer),
5′-GGGAATCCGAAGGTCCTGGT-3′ (antisense primer)
and GAPDH,
5′-CCTGGCCAAGGTCATCCATG-3′ (sense primer),
5′-GCCATGTAGGCCATGAGGTC-3′ (antisense primer)

resulting in PCR products of 293 bp and 519 bp respectively. GAPDH was used to normalize the NPP1 mRNA expression. The PCR conditions consisted of an initial cDNA synthesis reaction at 48 °C for 1 h, followed by a denaturing step for 2 min at 94 °C and 24 or 17 cycles of 1 min 94 °C, 1 min 63 °C and 1 min 72 °C for NPP1 and GAPDH respectively. After the last cycle, a final extension step was performed at 72 °C for 7 min. PCR was conducted in an Eppendorf Mastercycler gradient (VWR International, Belgium). Twenty microliters of PCR product were analyzed by agarose gel electrophoresis on a 1.5% (w/v) gel containing 0.5 μg/ml ethidium bromide. Bands were quantified with the Gel Doc 2000 system (Bio-Rad Laboratories, Belgium) and analyzed with Image Quant (Molecular Dynamics, Amersham Biosciences, USA).

2.5. Ecto-NPPase activity

C6 cells were cultivated in chemically defined medium as described above and stimulated for 48 h with 1 mM N^6–O'^2-dibutyryl cAMP (dbcAMP) or 5 μM (−)-isoproterenol. After a medium renewal, the ecto-NPPase assay was initiated by addition of 10 μM [γ-^{32}P] ATP to the extracellular medium. After incubation at 37 °C and 5% CO_2 for 90 min or at the time points indicated in the legends, medium was removed and hydrolysis stopped by addition of 100 mM EDTA. One μl of each sample was analyzed by TLC on polyethyleneimine cellulose plates (Merck, Germany). TLC chromatography was performed in 750 mM KH_2PO_4 (pH 3.0). After chromatography, radioactive spots were visualized by phospho-imaging and quantified using the ImageQuant software (PhosphoImager SI, Amersham Pharmacia Biotech, USA). The rate of ATP hydrolysis was calculated from its linear decrease as a function of time.

2.6. Quantification of transcription factors of the AP-1 family

C6 cells were incubated with 1 mM dbcAMP or 5 μM (−)-isoproterenol for 48 h. Nuclear extracts were prepared with the Nuclear Extract Kit according to the manufacturer's instructions and protein concentrations

were measured with the BCA protein assay. Transcription factors of the AP-1 family (phospho-c-Jun, c-Fos, FosB, Fra-1, Fra-2, JunD and JunB) were quantified in the nuclear extracts by TransAM AP-1 kit, which contains a 96-well plate on which a TPA responsive element (TRE) (5′-TGAGTCA-3′) has been immobilized. Primary antibodies recognize accessible epitopes of *c-fos*, FosB, Fra-1, Fra-2, *c-jun*, JunB or JunD proteins. Addition of a secondary HRP-conjugated antibody provides a sensitive colorimetric readout quantified by spectrophotometry at 450 nm.

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared with the Nuclear Extract Kit and protein concentrations were measured with BCA protein assay. A double-stranded oligonucleotide probe containing the AP-1 consensus-binding motif (5′-TTGGTAAGCTGACTCAGTGGTGATGAGAAT-3′) of the rat NPP1 gene was labelled at the 3′-end with biotin (Eurogentec, Seraing, Belgium). Samples were prepared with a lightshift chemiluminescent EMSA kit (Pierce, Aalst, Belgium) by addition of ultrapure water to yield 20 µl total sample volume, 2 µl 10× binding buffer (100 mM Tris, 500 mM KCl, and 10 mM DDT; pH 7.5), 1 µl polydI·dC, 1 µl 50% (v/v) glycerol, 1 µl 1% (v/v) NP-40, 1 µl 1 M KCl, 1 µl 100 mM $MgCl_2$, 1 µl 200 mM EDTA; pH 8.0 and 4 pmol unlabeled probe. To identify DNA binding proteins, 20 µg nuclear extract was incubated with 2 µg of antibodies to c-jun, c-fos, Fra-1, Fra-2, JunD and JunB (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) on ice for 1 h, before addition of 20 fmol biotin-labelled probe. Protein–DNA complexes were electrophoresed at 4 °C on a non-denaturating 5.5% (w/v) polyacrylamide gel in buffer containing 45 mM Tris, 45 mM boric acid, and 1 mM EDTA (pH 8.3) (5× TBE buffer). After transfer of the samples to a nylon membrane at 100 V for 30 min and at 4 °C, the membrane was UV-irradiated for 15 min. Biotin-labelled DNA was detected by the chemiluminescent nucleic acid detection module (Pierce, Aalst, Belgium) containing an enhanced luminol substrate for horseradish peroxidase (HRP) and according to manufacturer's instructions. The membrane was exposed to X-ray film for 2–5 min and the film was developed according to the manufacturer's instructions.

2.8. Statistical analysis

Results were represented as the mean ± standard error of the mean (S.E.M.) calculated from at least three independent experiments. The statistical significance of differences was calculated using the Student's t-test.

3. Results

3.1. Cyclic AMP-dependent down regulation of NPP1

Differentiation of C6 cells is induced by elevation of cAMP with membrane permeable cAMP-analogues such as dbcAMP or by a β-adrenoceptor-mediated activation of adenylate cyclase after stimulation of the cells with (−)-isoproterenol (Roymans et al., 2001). Cells were stimulated with increasing concentrations of dbcAMP and (−)-isoproterenol. After 48 h, cells were analyzed by RT-PCR and Western blotting for the presence of NPP1 mRNA and protein. Results show a dose-dependent down regulated expression of NPP1 mRNA and protein (Fig. 1). IC50-values for dbcAMP and (−)-isoproterenol of approximately 100 ± 30 µM and 150 ± 20 nM respectively were derived, indicating that the receptor-mediated down regulation of NPP1 is more effective. A maximal reduction in NPP1 mRNA and protein expression of at least 80 ± 3% and 75 ± 3% respectively, could be obtained after stimulation with 1 mM dbcAMP for 48 h, while 5 µM (−)-isoproterenol decreased NPP1 mRNA and protein expression by 75 ± 0.5% and 81 ± 1%, respectively.

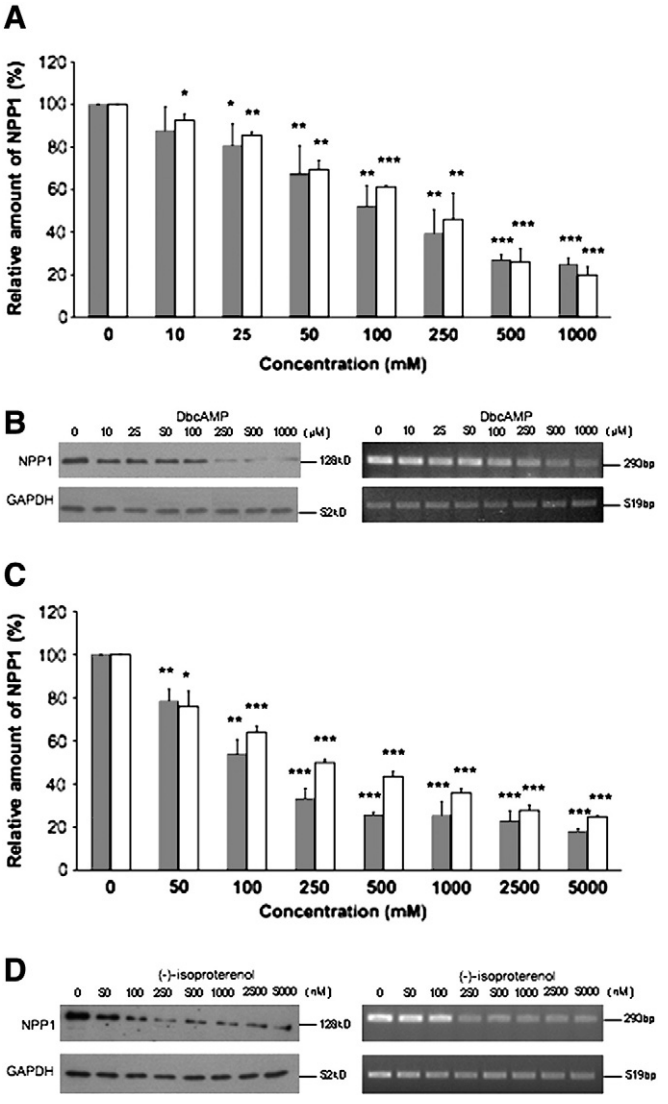

Fig. 1. Cyclic AMP-dependent down regulation of NPP1. C6 cells were stimulated with the indicated concentrations of dbcAMP (A, B) or (−)-isoproterenol (C, D). After incubation for 48 h, cells were harvested and analyzed for NPP1 protein (■) and mRNA (□) content by immunoblotting and RT-PCR, respectively. Results are expressed as the relative amount of NPP1 calculated from the NPP1/GAPDH ratio. The NPP1 content in the absence of dbcAMP or (−)-isoproterenol is taken as 100%. Panels B and D show an immunoblot (left panel) and an RT-PCR gel (right panel) representative for at least three independent experiments. P-values (***P<0.001; **P<0.01; *P<0.05) are the statistically significant difference between controls and cells stimulated with (−)-isoproterenol or dbcAMP.

Fig. 2 shows the time-dependent decrease of NPP1 mRNA and protein after stimulation with 1 mM dbcAMP or 5 µM (−)-isoproterenol. At the indicated time points, samples were analyzed by Western blotting and RT-PCR. After 72 h, dbcAMP stimulation decreased the NPP1 mRNA and protein expression by 74 ± 5% and 85 ± 5% respectively and (−)-isoproterenol decreased the mRNA and protein expression by 79 ± 4% and 80 ± 6% respectively. In both cases, down regulation is time dependent and levels off after 24 h of stimulation. Maximal down regulation of NPP1 mRNA and protein was observed after 24 h and 48 h of stimulation respectively.

As well as NPP1 mRNA and protein, the NPPase activity was also measured. In C6 cells ATP is mainly hydrolyzed by an ecto-NPPase. The rate of ATP hydrolysis into AMP and PP_i was calculated from the linear part of the ATP degradation into PP_i as a function of time. A decrease in ecto-NPPase activity was also observed after 48 h stimulation with 5 µM (−)-isoproterenol or 1 mM dbcAMP (Fig. 3). Autoradiography showed a reduction in PP_i formation upon stimulation of the cells with

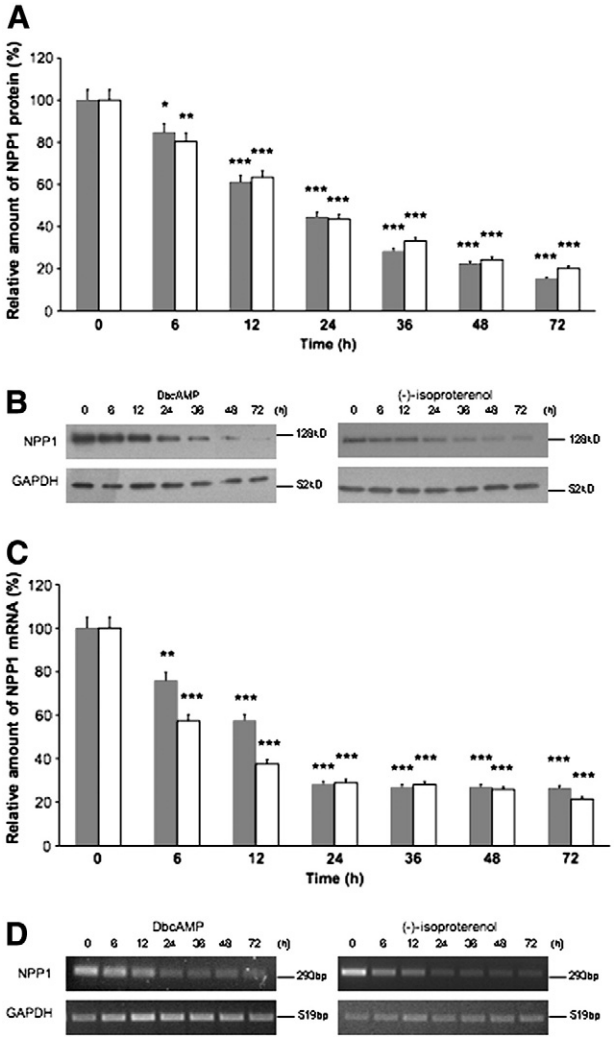

Fig. 2. Cyclic AMP-dependent down regulation of NPP1 as a function of time. C6 cells were stimulated with 1 mM dbcAMP (■) or 5 μM (−)-isoproterenol (□). At the indicated time points, cells were harvested and analyzed for NPP1 protein (A, B) and mRNA (C, D) content by immunoblotting and RT-PCR respectively. Results are expressed as the relative amount of NPP1 calculated from the NPP1/GAPDH ratio. The NPP1 content at 0 h is taken as 100%. The inserts show an immunoblot (B) and an RT-PCR gel (D) representative for at least three independent experiments. P-values (***P<0.001; **P<0.01; *P<0.05) are the statistically significant difference between controls and cells stimulated with (−)-isoproterenol or dbcAMP.

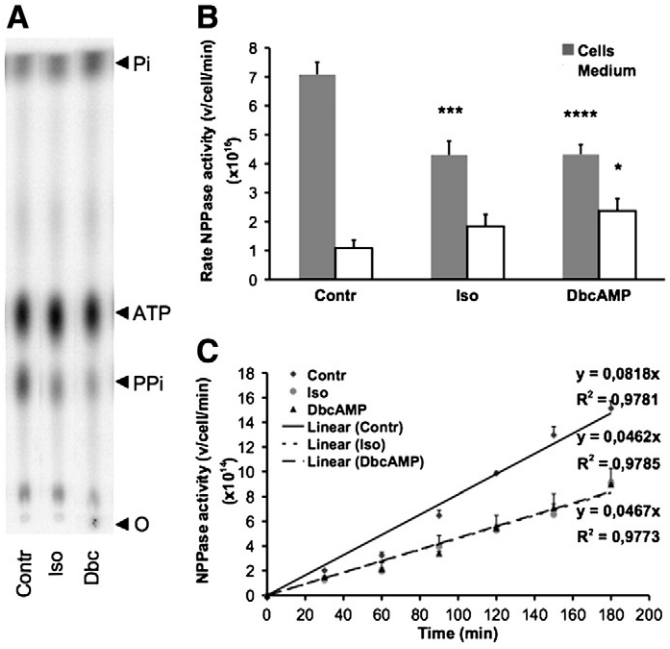

Fig. 3. Down regulation of ecto-NPPase activity by cAMP. C6 cells were grown in chemically defined medium up to a density of approximately 10^5 cells/cm^2. The ecto-NPPase activity was measured after stimulation of the cells with 1 mM dbcAMP or 5 μM (−)-isoproterenol. A. After 48 h incubation, 10 μM [γ-^{32}P]-ATP was added to the extracellular medium of the cells for 90 min. The reaction was stopped by addition of 100 mM EDTA and the medium analyzed by TLC. The autoradiograph shows the TLC plate of control cells (Contr) and cells stimulated with (−)-isoproterenol (Iso) or dbcAMP (Dbc), which is quantified by phospho-imaging. O, origin. B. The ecto-NPPase activity of NPP1 on cells and medium was determined from the rate per cell (v/cell) of ATP hydrolysis into AMP and PP_i. Radioactive spots were visualized by autoradiography and the amount of [γ-^{32}P]-ATP, PP_i and P_i was measured by phospho-imaging. C. ATP hydrolysis as a function of time. Stimulated and unstimulated cells were incubated with 10 μM [γ-^{32}P]-ATP. At the indicated time points samples were taken from the medium and the hydrolysis of ATP stopped by addition of 100 mM EDTA. Samples were analyzed by TLC and radioactive spots were visualized by autoradiography. Data are the mean ± S.E.M. determined from at least three independent experiments. The statistically significant difference from the hydrolysis rate in control cells is indicated (****P<0.0001; ***P<0.001; *P<0.05).

(−)-isoproterenol or dbcAMP (Fig. 3A). The NPPase activity of cells was reduced by 39.3 ± 11.1% and 39.0 ± 7.8% after a 90 min incubation of the cells with (−)-isoproterenol or dbcAMP respectively. In comparison, the soluble ecto-NPPase activity measured in conditioned medium of the cells only slightly increased, indicating that the decrease in membrane-bound NPPase activity is not explained by release of membrane-bound enzyme into the medium (Fig. 3B). The total NPPase activity was reduced with 43.5 ± 2.2% and 42.9 ± 2.3% after stimulation of the cells with (−)-isoproterenol or dbcAMP respectively (Fig. 3C).

3.2. The PI 3-K/PKB signalling pathway is not involved in NPP1 down regulation

In comparison with control cells, stimulation of C6 cells with 5 μM (−)-isoproterenol or 1 mM dbcAMP causes inhibition of NPP1 expression, whereas GFAP expression is up regulated more than twofold (Fig. 4).

To evaluate if the down regulation of the NPP1 protein is correlated with an up regulated GFAP synthesis, specific inhibitors were used for protein kinases that are involved in the differentiation-related signal transduction pathways (Fig. 5). C6 cells were stimulated with 5 μM (−)-isoproterenol in the presence or absence of H89 (1 μM), a membrane-permeable inhibitor of PKA or LY294002 (10 μM), a PI 3-K inhibitor. After a 1 h pre-incubation with one of these protein kinase inhibitors, cells were stimulated with 5 μM (−)-isoproterenol for 48 h. Neither inhibition of PKA nor PI 3-K abolished the decrease in NPP1 expression after (−)-isoproterenol stimulation (Fig. 6). In addition, GFAP expression was inhibited by at least 45 ± 10% after induction of differentiation in cells pre-treated with 10 μM LY294002 (Fig. 6). No effect on GFAP expression was observed when C6 cells were treated with PKA inhibitor. These data indicate that the cAMP-dependent inhibition of NPP1 expression does not require PI 3-K, which is an essential protein kinase for induction of GFAP.

As PI 3-K, an activator of PKB, is not involved in the down regulation of NPP1, it can be assumed that also PKB is not involved. Wang et al. (2001) demonstrated that the inactivation of PKB in C6 cells is caused by inhibition of Rap1. Cyclic AMP activates Rap1 through the Epac family of proteins, a recently discovered family of cAMP-binding nucleotide exchange factors, also known as cAMP GEFs, that catalyse GTP/GDP exchange on Rap1 (de Rooij et al., 1998; Van Kolen and Slegers, 2006). To investigate a possible involvement of a cAMP/Epac/Rap1/PKB-signalling pathway, C6 cells were stimulated for 48 h with 8-pCPT-2′-O-Me-cAMP, a specific membrane permeable activator of Epac. The basal expression of NPP1 was unaffected by treatment with 10, 100 and 500 μM of 8-pCPT-2′-O-Me-cAMP (data

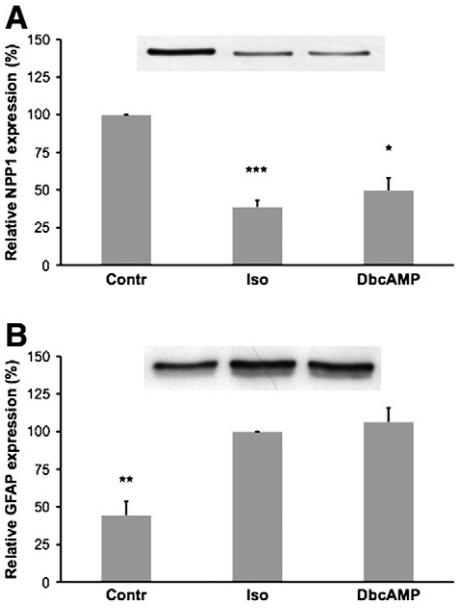

Fig. 4. The effect of cAMP on the expression of NPP1 and GFAP. Cells were stimulated with 5 μM (−)-isoproterenol (Iso) or 1 mM dbcAMP for 48 h. A. Relative NPP1 expression (%) with the control cells (Contr) taken as 100%. P-values (***$P<0.001$; *$P<0.05$) demonstrate the statistically significant difference in NPP1 expression between control cells and (−)-isoproterenol or dbcAMP stimulated cells. B. Relative GFAP expression with (−)-isoproterenol (Iso) stimulated cells taken as 100%. The statistically significant difference in relative GFAP expression is indicated (**$P<0.01$). Immunoblot intensities were quantified with Image Quant software. The insert shows an immunoblot representative for at least three independent experiments. All data are the mean ± S.E.M. determined from at least three independent experiments.

not shown). In addition, inhibition of GSK-3, a downstream effector of PKB, with different concentrations of lithium chloride (LiCl), a non-specific inhibitor of GSK-3, could not neutralize the inhibition of NPP1 after (−)-isoproterenol stimulation (data not shown).

Ca^{2+} is also required for PKB activity. To investigate the Ca^{2+}-dependency of the NPP1 pathway, cells were stimulated for 48 h with 50 μM BAPTA-AM, an intracellular Ca^{2+}-chelator. No alteration in NPP1 expression was observed indicating that the regulation of NPP1 expression does not require Ca^{2+} (Fig. 7A). As Ca^{2+} is also necessary for activation of PKB (Van Kolen and Slegers, 2004), the expression of NPP1 is independent of the PI 3-K/PKB signalling.

Furthermore, if a PKB phosphatase is the target of cAMP, inhibition of its activity should render PKB resistant to cAMP-dependent down regulation of NPP1. Treatment of C6 cells with 10 nM okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A), did not have an effect on NPP1 expression (Fig. 7B). This suggests that protein phosphatase PP2A, a downstream effector of Rap1, also did not play a role in the regulation of NPP1 expression.

3.3. NPP1 expression is independent of MAP kinase pathways

The involvement of MAP kinase pathways was investigated by stimulation of C6 cells with PD98059 (5 μM), a MEK-inhibitor, SB203580 (5 μM), an inhibitor for the p38-MAP kinase pathway and SP600125 (1 μM), an inhibitor of the JNK pathway (Fig. 5). After pre-incubation for 1 h, C6 cells were stimulated with 5 μM (−)-isoproterenol for 48 h. The basal expression of NPP1 was unchanged after addition of the three MAP kinase inhibitors in comparison with untreated cells (Fig. 7C). In addition, combination of the inhibitors with (−)-isoproterenol did not neutralize the inhibition of NPP1 expression. Hence, no evidence could be found that MAP kinases play a role in the regulation of NPP1 expression.

3.4. NPP1 gene expression does not involve AP-1 transcription factors

Analysis of the rat NPP1 gene promoter indicated a putative AP-1 consensus sequence (−1609) 5′-TGACTCA-3′ (−1603). To determine which AP-1 binding transcription factor could complex to this consensus sequence, a combination of ELISA and electrophoretic mobility shift assays (EMSA) were used. First, it was investigated whether cAMP modified the expression of transcription factors of the

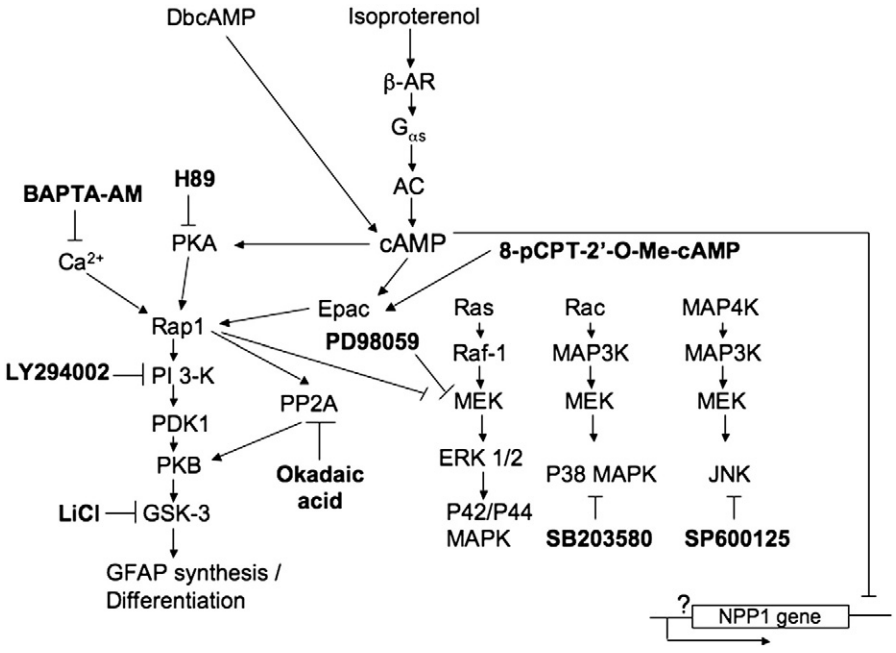

Fig. 5. Cyclic AMP-mediated signalling mechanism in C6 cells. Activators or inhibitors used in this study to identify the signalling pathway that regulates the GFAP and NPP1 gene expression are printed in bold (β-AR, β-adrenoceptor; Gαs, Gα subunit of a stimulatory G protein; PKA, protein kinase A; AC, adenylate cyclase; PI 3-K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide-dependent kinase-1; PKB, protein kinase B; GSK-3, glycogen synthase kinase-3; PP2A, protein phosphatase 2A; Epac, exchange protein activated by cAMP; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; JNK, c-Jun N-terminal kinase).

AP-1 family by an ELISA-based TransAM transcription assay kit. The specificity of the assay was confirmed by the use of a soluble wild-type AP-1 consensus oligonucleotide preventing AP-1 transcription factor binding to the probe immobilised on the plate. A soluble mutated consensus oligonucleotide had no effect on AP-1 binding. Nuclear extract of WI-38 cells stimulated by TPA was used as positive control for phospho-*c-jun* (p-*c-jun*), *c-fos*, FosB, Fra-1, Fra-2, JunB and JunD. In nuclear extracts of unstimulated C6 cells, p-*c-jun*, *c-fos*, Fra-1, JunB and JunD were the factors that mainly bound to the used AP-1 consensus sequence (Fig. 8). Extracts of cells stimulated with dbcAMP and (−)-isoproterenol showed a significantly decreased amount of p-*c-jun* bound to the AP-1 probe, whereas the amount of *c-fos* significantly increased. In addition, it was also observed that the amount of JunD, bound to the AP-1 probe, significantly decreased after stimulation of the cells with (−)-isoproterenol (Fig. 8). Cyclic AMP stimulation did not change the amount of FosB, Fra-1, Fra-2 and JunB bound to the AP-1 probe. These results suggest that inhibition of the AP-1 transcriptional activity could be involved in the regulation of NPP1 expression. However, this supposition was not confirmed by EMSA in which nuclear extract of C6 cells was added in a binding reaction solution together with the biotin-labelled target DNA that contains the AP-1 consensus sequence. Based on the fact that a DNA–protein complex migrates slower than non-bound DNA, a "shift" in the labelled DNA band was expected. However, EMSA did not produce a supershifted DNA-binding complex combined with any of the antibodies (data not shown), indicating that none of the assayed members of the AP-1 transcription factor family interacted with the consensus-binding site present in the NPP1 promoter sequence.

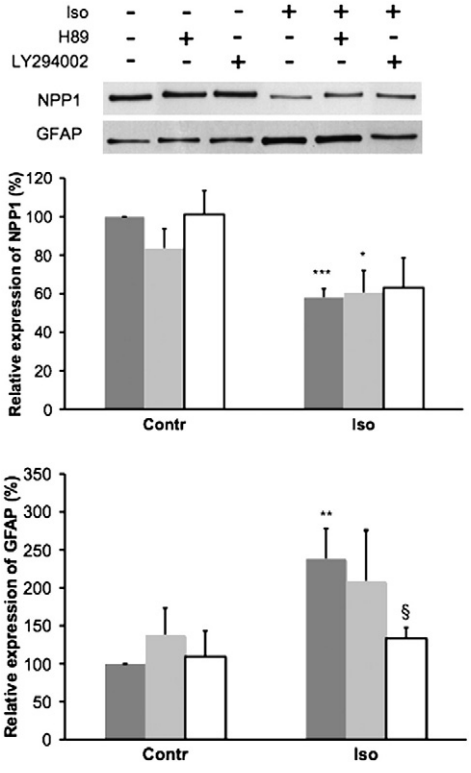

Fig. 6. Effect of protein kinase inhibitors on the expression of NPP1. Cells were pre-incubated for 1 h with H89 (1 μM, ■) and LY294002 (10 μM, □) to inhibit PKA and PI 3-K, respectively. After 1 h, cells were stimulated with (−)-isoproterenol (5 μM) for 48 h. The amount of NPP1 protein was measured by immunoblotting. The amount in control cells is taken as 100%. The data are represented as mean ± S.E.M. of at least three independent experiments. Statistically significant differences from control cells (*) and from (−)-isoproterenol stimulated cells ($) are indicated (***P<0.001; **P<0.01; *P<0.05; $P<0.05).

4. Discussion

C6 cells have a low basal adenylyl cyclase activity despite an efficient coupling of the enzyme to β-adrenoceptors (Valeins et al., 1992). The addition of agents such as (−)-isoproterenol or dbcAMP results in an increase in intracellular concentrations of cAMP, which is partially extruded by a unidirectional efflux pump (Henderson and Strauss, 1991). Elevation of cAMP induces differentiation of C6 cells, characterized by inhibition of cell growth, activation of GFAP synthesis and a change in shape from a bipolar to a process-bearing morphology. This process is independent of PKA activation (Messens and Slegers, 1992; Anciaux et al., 1997; Roymans et al., 2000).

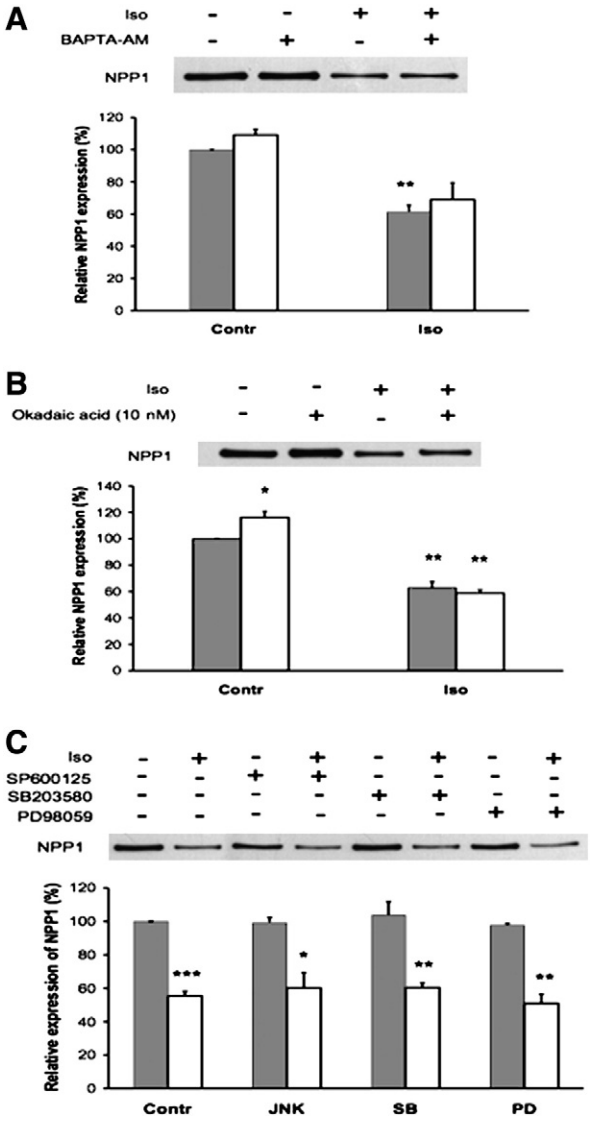

Fig. 7. Effect of BAPTA-AM, okadaic acid and MAP kinase inhibitors on the expression of NPP1. A. Cells were pre-incubated without (■) or with (□) BAPTA-AM (50 μM). After 1 h incubation, cells were non-stimulated (Contr) or stimulated with (−)-isoproterenol (5 μM, Iso) for 48 h. B. Cells were incubated without (■) or with (□) 10 nM okadaic acid. Subsequently, cells were stimulated with (−)-isoproterenol as in A. C. Cells were pre-incubated with the JNK inhibitor SP600125 (1 μM, SP), the p38-MAP kinase inhibitor SB203580 (5 μM, SB) or the MEK inhibitor PD98059 (5 μM, PD). Subsequently, cells were non-stimulated (■) or stimulated with (−)-isoproterenol (5 μM, Iso, □) as in A. The amount of NPP1 protein was measured by immunoblotting. The amount in cells not stimulated with (−)-isoproterenol (Contr) is taken as 100%. P-values (***P<0.001, **P<0.01, and *P<0.05) are the statistically significant difference between controls and cells stimulated with (−)-isoproterenol. All results are represented as the mean ± S.E.M. of at least three independent experiments.

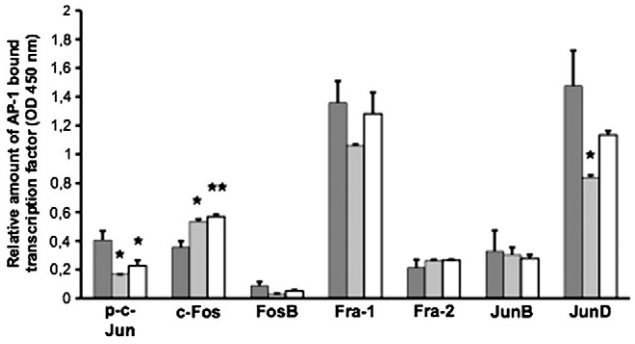

Fig. 8. Transcription factors of the AP-1 family in C6 cells. Cells were non-stimulated (■) or stimulated with (−)-isoproterenol (5 μM, Iso, ▒) or dbcAMP (1 mM, □). After 48 h of incubation nuclear extracts were prepared as described in Materials and methods. The amount of AP-1 bound transcription factor was measured using a TransAM kit, based on the binding to an immobilized TPA-response element. Bound factors were detected with specific antibodies and the amount quantified by spectrophotometry at 450 nm using a secondary HRP-conjugated antibody. Data represent the mean ± S.E.M. of at least three independent experiments. P-values (**$P<0.01$ and *$P<0.05$) are the statistically significant difference compared with not stimulated cells.

In this communication, we demonstrate that an increase in intracellular cAMP-concentration down regulates the levels of NPP1 mRNA, NPP1 protein and the ecto-NPPase activity in C6 cells. DbcAMP or (−)-isoproterenol inhibits NPP1 expression in a time and dose-dependent manner. Although the soluble ecto-NPPase activity slightly increases, it can be assumed that no shedding into the medium occurs by cleavage of the extracellular part of the protein containing the phosphodiesterase activity. Using an antibody raised against the intracellular part of the NPP1 protein, it was shown that NPP1 down regulation was not the result of extracellular cleavage.

We also demonstrate by the use of H89, an ATP-site directed inhibitor of PKA, that the cAMP-dependent effect on NPP1 expression is not dependent on PKA activation. Although most of the actions of cAMP are exerted through PKA, some actions have been reported to be independent of PKA and are transduced to PKB via Epac (Mei et al., 2002; Stork and Schmitt, 2002; Morel et al., 2005). Binding of cAMP to Epac induces its GEF activity towards Rap1 (de Rooij et al., 1998; Fuld et al., 2005). However activation of Epac by 8-pCPT-2′-O-Me-cAMP in C6 cells could not demonstrate an involvement of Epac in the cAMP-dependent down regulation of NPP1. Previously we have shown that differentiation induced by cAMP, characterized by an increase in GFAP expression and by morphological changes, is due to inhibition of PKB although PI 3-K activity is required (Roymans et al., 2001; Van Kolen and Slegers, 2004). The data with the PI 3-K inhibitor also indicate that inhibition of NPP1 expression is not active in the morphological changes of C6 cells upon dbcAMP or (−)-isoproterenol stimulation. In contrast, an increase in cAMP inhibits the expression of NPP1 by a signalling pathway that is different from the pathway that induces GFAP expression. Indeed, PI 3-K, a protein kinase required for the induction of GFAP, is not functional in the inhibition of NPP1 gene expression.

In C6 cells, Wang et al. demonstrated that cAMP-dependent inhibition of PKB is due to inactivation of Rap1 by a pathway that is independent of PKA or Epac (Wang et al., 2001). PKB is a downstream effector of cAMP-mediated activation of Rap1 and cAMP elevation results in PKB inactivation. On the contrary, in PCCL3 thyroid cells Hong et al. (2008) demonstrated that constitutively active PKB, by substitution of Ser^{473} and Thr^{308} (the activating phosphorylation sites), is resistant to cAMP inhibition, suggesting the action of a phosphatase in these cells. They demonstrated that Epac- and PKA-specific analogues synergistically inhibit PKB and showed that Epac is associated in a complex with PKB and PP2A where the phosphatase activity is positively modulated by cAMP in a PKA- and Rap1-dependent manner. They identified a novel cAMP/Epac/PKA/Rap1b/PP2A-signalling module that regulates PKB phosphorylation (Hong et al., 2008). It has also been shown that *in vitro* PKB is inactivated by PP2A and is stimulated in cells upon treatment with okadaic acid (Andjelković et al., 1996). Our data shows no change in NPP1 expression following PP2A inhibition after treatment of C6 cells with okadaic acid.

The downstream effector of PKB, GSK-3, has a central position in the regulation of the activity of several transcription factors (Ferkey and Kimelman, 2000; Kim et al., 2001). However inhibition of GSK-3 with LiCl did not affect the cAMP-dependent down regulation of NPP1. It has been reported that PKB activation requires Ca^{2+}-ions and calmodulin. Pre-incubation of C6 cells with the Ca^{2+}-chelator BAPTA-AM resulted in a complete inhibition of the phosphorylation of PKB (Van Kolen and Slegers, 2004) but had no influence on the expression of NPP1. Afterwards stimulation of C6 cells with 5 μM (−)-isoproterenol did not change the down regulation in NPP1. This confirms that PKB has no function in the down regulation of NPP1.

It has been reported that cAMP inhibits MAP kinase activity in both primary astrocytes and C6 cells (Kurino et al., 1996; Qiu et al., 2000). It has been suggested that this effect depends on Ras/Raf-1 signalling, which inhibits MAP kinase, or Rap1/B-Raf signalling, which activates MAP kinase, depending on the cell type (Kurino et al., 1996; Vossler et al., 1997; Dugan et al., 1999; Qiu et al., 2000). It is unlikely that MAP kinases are active in the down regulation of NPP1 since the MAP kinase-inhibitors PD98059, SB203580 and SP600125 remain ineffective.

Promoter analysis and prediction of potential transcription factor binding sites is often used to identify cellular regulatory networks. Several potential binding sites for specific transcription factors were identified in the promoter of rat NPP1 including a binding site for AP-1 at nucleotides −1609 to −1603 (TGACTCA). Studies have demonstrated that Fos and Jun serve as transcriptional factors in the expression of various genes through interaction with AP-1 and AP-1-like DNA domains in their promoter regions. These transcription factors appear to be important participants in ligand-mediated signal transduction pathways. Our results are in line with previous studies in C6 cells where treatment with the β-adrenergic agonist, (−)-isoproterenol, results in an increase in *c-fos* mRNA and a decrease of *c-jun* mRNA level (Gubits and Yu, 1991; Gubits et al., 1992; Ye et al., 1994; Zhang et al., 2000; Lee et al., 2001). In addition, studies also show that the activation of adenylate cyclase by forskolin appears to down regulate *c-jun* mRNA expression by stimulation of PKA in C6 cells (Lee et al., 1999). However, in breast cancer cells and prostate tissue, *c-jun* expression was increased after activation of PKA (Lacroix and Body, 1997; Murtha et al., 1997). It has also been demonstrated that *c-jun* levels are differentially regulated in neurons and glial cells. An increase in cAMP, by treatment with forskolin or by an increase in intracellular calcium levels, down regulated *c-jun* expression in glial cells, but not in neurons (De Felipe and Hunt, 1994). JunD is constitutively expressed in C6 cells and its RNA level decreased after treatment with forskolin. This agent also induces c-Fos and JunB (Kobierski et al., 1991). This is contradictory with other studies that failed to detect induction of JunB after stimulation of neurons and glial cells with cAMP analogues or forskolin, suggesting that such mechanisms may not operate in these cells (De Felipe and Hunt, 1994). In agreement with Kobierski et al. (1991), in C6 cells, we observed a significant level of JunD, which is decreased upon treatment of the cells with (−)-isoproterenol. Our results show no change in JunB expression in dbcAMP or (−)-isoproterenol stimulated cells.

AP-1 proteins are involved in cAMP-dependent gene expression as regulators of cAMP responsive element (CRE), which is a point of convergence for many extracellular and intracellular signals, including cAMP, calcium, G-protein coupled receptors, as well as the CRE transcriptional response element (TRE) (Kobierski et al., 1991). The promoter region of the rat NPP1 gene contains an AP-1 consensus element. However, although cAMP activated some members of the AP-1 family, this activation is not involved in the regulation of the NPP1 gene. In fact, AP-1 has been shown to regulate the cAMP-dependent differentiation process without regulation of the NPP1 gene. Besides

an AP-1 consensus sequence, several other motifs are present in the promoter of the NPP1 gene which bind cAMP regulated transcription factors such as sp1, ATF6, NFAT, Egr-1, and C/EBP. It remains to be determined which transcription factors are involved in the cAMP-dependent inhibition of NPP1 gene expression.

In conclusion, cyclic AMP-mediated induction of differentiation increases GFAP synthesis but inhibits the expression of NPP1 by different signalling pathways. Further research is needed to identify the cAMP-dependent cascade involved in NPP1 protein down regulation in C6 cells. Elucidation of the mechanisms that regulate the expression of NPP1 protein could lead to new therapies or the design of drugs that normalize E-NPP expression in disorders associated with pathologic bone calcification (Terkeltaub, 2006), type 2 diabetes (Abate et al., 2006) and tumour progression (Aerts et al., in press), where an aberrant expression of NPP1 is observed.

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