European Journal of Pharmacology 659 (2011) 1–6

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

NO-induced activation of cyclic GMP-dependent pathway down regulates ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) protein in rat C6 glioma

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ARTICLE INFO

Article history:
Received 7 September 2010
Received in revised form 31 January 2011
Accepted 1 March 2011
Available online 15 March 2011

Keywords:
NPP1
C6 glioma cells
Cyclic GMP
Nitric oxide

ABSTRACT

In rat C6 glioma cells, the ecto-nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1), a modulator of purinergic receptor signaling, is down regulated after an increase in intracellular cAMP by addition of dibutyryl cAMP, a membrane-permeable cAMP-analog, or by activation of the β-adrenoceptor receptor with (−)-isoproterenol (Aerts et al., 2011, Eur. J. Pharmacol. 654, 1–9). In this communication we studied the effect of nitric oxide (NO)/cGMP, a pathway also affecting purinergic receptor signaling, on the level of NPP1 protein. Sodium nitroprusside (SNP), a NO donor, reduces NPP1 protein in a dose-dependent manner. A combination of SNP and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, an inhibitor of soluble guanylate cyclase, demonstrated that NO-dependent down regulation of NPP1 was caused by NO-sensitive guanylyl cyclase. Treatment with Rp-*p*CPT-cGMPS, an inhibitor of protein kinase G (PKG), showed that PKG is not involved in the down regulation of NPP1. In addition, we have shown that the cAMP- and cGMP-dependent decrease in NPP1 expression is unrelated. These results indicate that NO/cGMP regulates the level of NPP1 protein by a pathway that differs from the cAMP-induced decrease in NPP1.

1. Introduction

Nitric oxide (NO) is a signaling molecule that affects a variety of physiological and biochemical processes including synaptic transmission, apoptosis, regulation of gene expression, post-translational modification of proteins (Bogdan, 2001; Blaise et al., 2005). In brain, NO is produced by neural, glial and endothelial cells and cGMP formation has been reported as the main NO activated pathway (Chung et al., 2001). NO modulates purinergic receptor signaling by induction of ATP from astrocytes (Bal-Price et al., 2002; Mehta et al., 2008). Cyclic GMP is generated by guanylyl cyclase (GC), which consists of a membrane-bound isoform also known as particulate guanylyl cyclase (pGC), which is activated by atrial, brain and C-type natriuretic peptides (ANP, BNP and CNP, respectively), and a soluble isoform (sGC) that is activated by NO. NO can be formed by nitric oxide synthases (NOS) such as: neuronal NOS (nNOS, NOS I), inducible NOS (iNOS, NOS II) and endothelial NOS (eNOS, NOS III) (Hanafy et al., 2001;Domek-Łopacińska and Strosznajder, 2005; Feil and Kemp-Harper, 2006).

The main effector of cGMP is cyclic nucleotide-dependent protein kinase (PKG), but it also influences cyclic nucleotide gated channels (CNG) and cyclic nucleotide-dependent phosphodiesterases (PDEs). The latter enzyme family (PDE1 to PDE11) plays a major role in cell signaling by hydrolyzing cAMP and cGMP (Lugnier, 2006; Bender and Beavo, 2006).

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. C6 cells generate cAMP from membrane-permeable cAMP analogs or in response to stimulation by agonists of the β-adrenoceptor that is positively coupled to adenylate cyclase. Although little is known about the cGMP-dependent signaling pathways in these cells, it has been reported that 5-hydroxytryptamine generates cGMP through a NO-dependent pathway (Kagaya et al., 1995). Eguchi et al. (1992) found that C6 cells also generate cGMP in response to natriuretic peptides.

Our laboratory has shown the presence of ecto-nucleotide pyrophosphatase (EC 3.6.1.9)/phosphodiesterase 1 (EC 3.1.4.1) (E-NPP1) on the plasma membrane of C6 cells (Grobben et al., 1999). NPP1 belongs to a multigene family with seven members. NPP1 and NPP3 are type II transmembrane glycoproteins with a short intracellular amino-terminal domain. NPP2 is not a transmembrane protein but is secreted after removal of the N-terminal signal peptide (Jansen et al., 2005). In addition, NPP4–7 are type I transmembrane proteins with a short intracellular carboxy-terminal domain. The extracellular domain of NPP1 contains a phosphodiesterase activity that hydrolyzes pyrophosphate or phosphodiester bonds in nucleotides and their derivatives. E-NPPs have multiple physiological roles and biochemical processes such

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doi:10.1016/j.ejphar.2011.03.004

as nucleotide recycling, modulation of purinergic and insulin receptor signaling, regulation of extracellular pyrophosphate levels, cell proliferation, apoptosis and stimulation of cell motility (Rathbone et al., 1999; Goding et al., 2003; Stefan et al., 2005; Claes and Slegers, 2004; Abate et al., 2006; Terkeltaub, 2006; Aerts et al., 2011a).

Recently, our laboratory demonstrated that a cAMP-dependent pathway down regulates the expression of NPP1 in C6 cells (Aerts et al., 2011a). The present study demonstrates that also the NO/cGMP pathway can regulate the level of NPP1. The NO/cGMP signaling mechanisms appear to be unrelated to the cAMP-dependent pathway.

2. Materials and methods

2.1. Materials

Nucleotide derivatives N^6-O'^2-dibutyryl cAMP (dbcAMP) and N^2-O'^2-dibutyryl cGMP (dbcGMP), (−)-isoproterenol, C-type natriuretic peptide (CNP), 3-(4-amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b] pyridine (BAY 41–2272), Rp-8-(4-chlorophenylthio) adenosine-3′, 5-cyclic monophosphorothioate (Rp-8-CPT-cAMPS) and the PDE inhibitor dipyridamole were from Sigma RBI (Köln, Germany). N (G)-nitro-L-arginine-methyl ester (L-NAME), sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ), Rp-isomer-8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphorothioate (Rp-*p*CPT-cGMPS), and 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis (aceto-methyl ester) (BAPTA-AM) were from Calbiochem (Merck Biosciences N.V., Overijse, Belgium). Polyclonal anti-NPP1 antibody was kindly provided by Dr. H. Sakoda (Sakoda et al., 1999).

2.2. Cell culture

Rat C6 glioma cells (ATCC no. CCL 107) were obtained from ATCC (Manassas, USA) (passage n°: 40–49) and were maintained in monolayer culture as described previously (Slegers and Joniau, 1996). Experiments were performed on 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 (GIBCO, Paisley, Scotland), and 30 nM sodium selenite (Sigma Chemical Co., St. Louis, USA). Culturing was in a humidified atmosphere of 5% CO_2 and at 37 °C. Cell numbers were measured in a hemocytometer 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 density of approximately 10^5 cells/cm^2. After stimulation and incubation for 48 h as described in the figure legends, the culture medium was removed and the cells dissolved in SDS-PAGE sample buffer (2×) [40 mM Tris–HCl (pH 6.8), 1% (w/v) SDS, 20% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 1 mM $NaVO_3$, 1 mM NaF, 30 mM DTT]. Samples were boiled for 5 min and an equal number 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 was detected with a polyclonal rabbit anti-NPP1 antibody (1:4000) as described previously (Sakoda et al., 1999). Following incubation of the nitrocellulose membrane with primary antibodies against NPP1, the blot was incubated with horseradish peroxidase-labeled anti-rabbit IgG (1:1000), as 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, Sunnyvale, USA).

2.4. 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 between means was calculated using the Student's *t*-test.

3. Results

3.1. Cyclic nucleotide-dependent down regulation of NPP1

Previous studies in our laboratory showed a down regulation in NPP1 expression after intracellular cAMP elevation by treatment with (−)-isoproterenol, a β-adrenoceptor agonist or by a membrane permeable cAMP-analog such as dibutyryl cAMP (Aerts et al., 2011a). In this communication we investigated the effect of the NO/cGMP pathway on the levels of NPP1 protein. Therefore, C6 cells were stimulated with 1 mM dbcGMP or with 1 μM CNP, an activator of pGC. A reduction of NPP1 protein expression of at least 51% ± 10% and 58% ± 3% was found after addition of (−)-isoproterenol and dbcAMP respectively (Fig. 1). DbcGMP also showed a reduction in NPP1 expression of at least 46% ± 4%, while CNP was without effect, suggesting that pGC is not involved in the regulation of NPP1 expression.

In subsequent experiments sodium nitroprusside (SNP) was used as NO-donor. SNP has been reported as an efficient inducer of cGMP in C6 cells (Vigne et al., 1993). SNP induces a $[Ca^{2+}]_i$ increase by enhanced Na^+–Ca^{2+} exchanger activity or by increasing eNOS activity, to produce cGMP in a Ca^{2+}/calmodulin-dependent manner (Amoroso et al., 2000; Sardón et al., 2004). Based on this observation, cells were incubated for 48 h with 50 μM BAPTA-AM, a well-known membrane permeable Ca^{2+}-chelator (Fig. 1). We observed no alteration in the amount of NPP1 indicating that Ca^{2+} is not required in the regulation of NPP1 expression.

Subsequently, we tested the involvement of sGC by treatment of the cells with increasing concentrations of SNP or SNAP, which are NO donors that cause cGMP-mediated sGC activation (Huitema et al., 2006). A dose-dependent down regulation of NPP1 was observed after treatment with increasing concentrations of SNP. A maximal effect

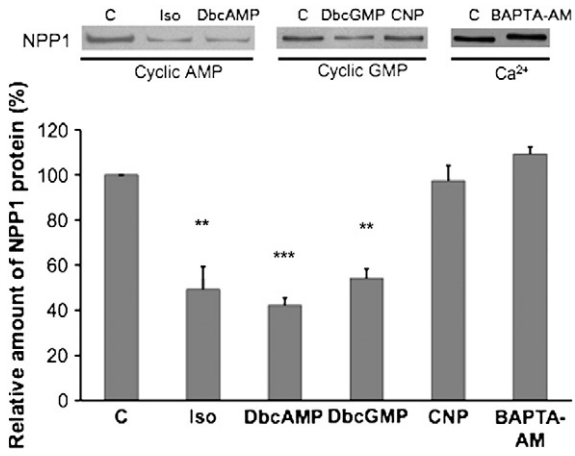

Fig. 1. Cyclic AMP and cGMP-dependent down regulation of the expression of NPP1. Cells were grown in chemically defined medium and were incubated for 48 h with (−)-isoproterenol (5 μM, Iso), dibutyryl cyclic AMP (1 mM, DbcAMP), dibutyryl cyclic GMP (1 mM, DbcGMP) and CNP (1 μM). To measure the effect of Ca^{2+} on the expression of NPP1, cells were incubated for 48 h with 50 μM BAPTA-AM. Cell numbers were counted with a hemocytometer and an equal number was used for analysis by immunoblotting. The immunoblots shown are representative of at least three independent measurements. The results are expressed as relative amount of NPP1 (%) with control cells (C) taken as 100%. P-values (*** $P<0.001$; ** $P<0.01$; * $P<0.05$) demonstrate the statistically significant difference in NPP1 expression between control cells and stimulated cells. All data are the mean ± S.E.M. of at least three independent experiments.

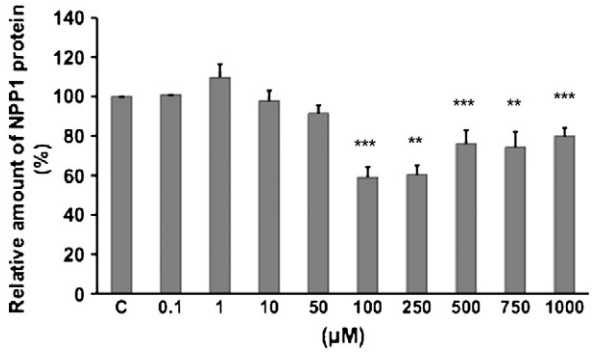

Fig. 2. Dose-dependent down regulation of NPP1 by SNP. Cells were grown in chemically defined medium and incubated for 48 h with the indicated concentrations of SNP. Subsequently, cells were harvested and analyzed for NPP1 protein by immunoblotting. Results are expressed as the relative amount of NPP1. The NPP1 content in the absence of SNP (C) was taken as 100%. The immunoblot shown is representative of at least three independent experiments. The statistically significant difference in relative NPP1 expression is indicated (** $P<0.01$; *** $P<0.001$).

was found at concentrations of 100 μM and 250 μM SNP, causing reduction of 41%±7% and 40%±8% respectively (Fig. 2). However, SNAP inhibited NPP1 expression only 10%±2% in the same concentration range (data not shown). Higher concentrations of SNP had a less pronounced effect, probably due to the effect of the iron-containing part of SNP that suppresses NO production by a protective effect on cell death (Amoroso et al., 2000; Harhaji et al., 2004).

3.2. NPP1 down regulation requires NO-induced activation of sGC but no PKG activity

C6 cells were treated with either ODQ, a potent and selective inhibitor of NO-sensitive GC, or BAY 41-2272, a NO-independent activator of sGC (Fig. 3). When cells were pre-incubated for 1 h with ODQ, an inhibitor of sGC, followed by addition of 100 μM SNP and incubation for 48 h, the amount of NPP1 returned to the basal level of control cells. Stimulation of cells for 48 h with 5 μM BAY 41-2272 did not affect the amount of NPP1. However, combined with 100 μM SNP, BAY41-2272 potentiated the down regulation of NPP1. This indicates that probably cGMP-dependent down regulation of NPP1 occurs through NO-dependent activation of sGC.

Rp-8-pCPT-cGMPS (Rp-cGMP) (100 μM), a cell permeable inhibitor of PKG was added for 1 h before stimulation of the cells with 100 μM SNP for 48 h or with PBS as a control (Fig. 4). Although cells treated with Rp-cGMP showed a small but significant increase in NPP1 protein, no marked difference could be observed when Rp-cGMP treatment was combined with addition of SNP, indicating that the NO induced down regulation of NPP1 is not dependent on PKG activity.

3.3. Cyclic AMP and cGMP-dependent down regulation of NPP1 are unrelated

Since cAMP as well as cGMP could cause a down regulation of the amount of NPP1, it is not unlikely that they activate signaling pathways that converge at a common effector involved in the regulation of NPP1 expression. Cells were treated with 100 μM Rp-8-CPT-cAMPS (Rp-cAMP), a selective inhibitor of PKA. After 1 h, cells were stimulated for 48 h with 100 μM SNP. As shown in Fig. 4, Rp-cAMP combined with SNP partially reversed the down regulation of NPP1 exerted by SNP, suggesting that PKA activation may have a negative effect on the NO/cGMP-dependent decrease in the amount of NPP1. Stimulation of cells with (−)-isoproterenol in the absence or presence of 100 μM Rp-cAMP, shows no difference between Rp-cAMP stimulated and non-stimulated cells (data not shown). Hence, inhibition of PKA by Rp-cAMP did not block the cAMP-dependent decrease in NPP1. These data are in line with previous studies of our laboratory, which showed that the cAMP-dependent down regulation of NPP1 expression occurs independently of PKA activation (Aerts et al., 2011a).

Also, inhibition of NOS, achieved by treatment of cells with L-NAME, a non-specific inhibitor of NOS, reduces the elevation of cGMP by NO. Therefore, cells were pre-incubated for 1 h with 10 μM L-NAME followed by treatment with 5 μM (−)-isoproterenol for 48 h. In

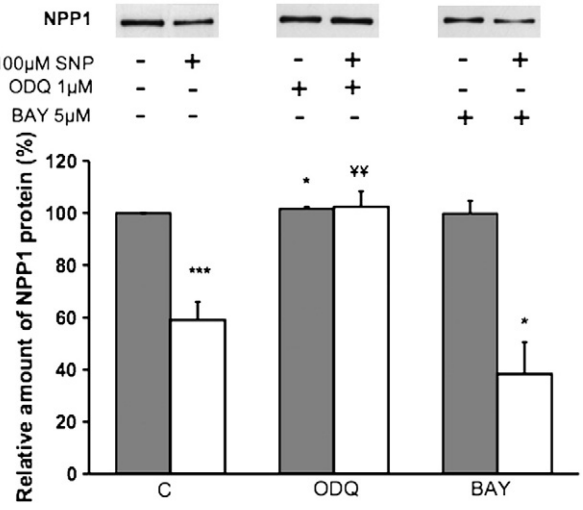

Fig. 3. Effect of BAY 41-2272 and ODQ on the expression of NPP1 in the presence of SNP. Cells were grown in chemically defined medium and pre-incubated for 1 h with 5 μM BAY 41-2272 or 1 μM ODQ (■). Subsequently, cells were stimulated with 100 μM SNP (□). Non-stimulated cells were incubated with PBS. After 48 h, cells were harvested and analyzed for NPP1 protein content by immunoblotting. Results are expressed as the relative amount of NPP1 and non-stimulated control cells were taken as 100%. The immunoblot shown is representative of at least three independent experiments. The statistically significant differences in relative amount of NPP1 protein between non-stimulated and stimulated cells are indicated (* $P<0.05$; ** $p<0.01$; *** $=P<0.001$ compared to the control group; ¥¥ $=P<0.01$ compared to the SNP-stimulated group).

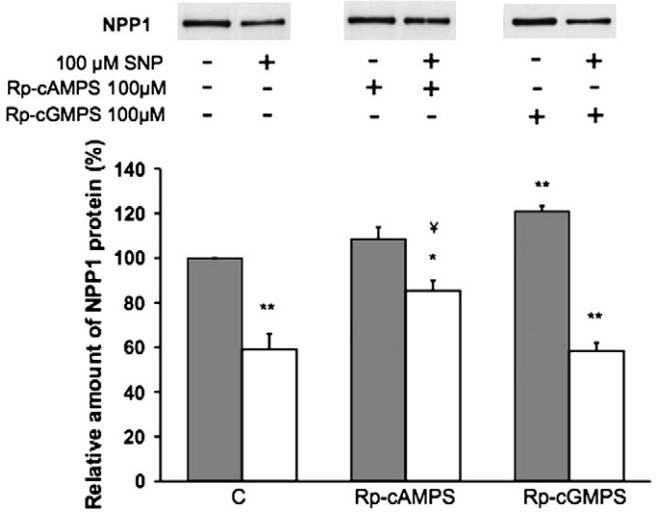

Fig. 4. Effect of Rp-*p*CPT-cGMPS (Rp-cGMP) and Rp-8-CPT-cAMPS (Rp-cAMP) on the expression of NPP1 in the presence of SNP. C6 cells were grown in chemically defined medium and were pre-incubated with 100 μM Rp-cGMP or 100 μM Rp-cAMP (■). After 1 h, cells were stimulated with 100 μM SNP (□). Non-stimulated cells were incubated with PBS. After 48 h incubation, the amount of NPP1 protein was measured by immunoblotting. Results are expressed as the relative amount of NPP1. Non-stimulated control cells (C) were taken as 100%. The immunoblot shown is representative of at least three independent experiments. The statistically significant differences in relative amount of NPP1 protein between non-stimulated and stimulated cells are indicated (* $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared to the control group; ¥ $=P<0.05$ compared to the SNP-stimulated group).

agreement with previous results NOS inhibition with L-NAME had no effect on the inhibition of NPP1 expression by cAMP (data not shown).

Dipyridamole inhibits mainly cGMP-dependent PDE 5 but is also a weak inhibitor of cAMP-PDEs. We examined the effect of this PDE inhibitor on the expression of NPP1. C6 cells were incubated for 48 h with different concentration of dipyridamole. Dipyridamole (10 μM) showed a maximal decrease in the amount of NPP1 protein (Fig. 5). These data are in agreement with experiments on the cGMP-dependent down regulation of NPP1.

4. Discussion

Physiological roles related to brain development have been associated with purinergic signaling (Cognato Gde et al., 2008). NPP1 and NPP2 of E-NPP family are capable of hydrolyzing various nucleotides and are functional in purinergic receptor signaling by hydrolyzing ATP into AMP and PPi. AMP is subsequently converted into adenosine by ecto-5′-nucleotidases, that has different effects depending on the cell type (Goding et al., 2003). In C6 glioma cells, adenine nucleotides, especially AMP and adenosine, inhibit the growth stimulation induced by activation of P2Y receptors by ATP (Claes et al., 2001). Castillo et al. reported that these cells express A1 and A2 adenosine receptors, which are functionally coupled to adenylate cyclase inhibition and stimulation respectively (Castillo et al., 2007).

The NO/cGMP pathway stimulates the release of ATP in astrocytes and modulates glia-neuron crosstalk by affecting purinergic receptor signaling in brain (Bal-Price et al., 2002; Mehta et al., 2008). In addition, Isakovic et al. (2008) suggested that adenosine-mediated inhibition of glial NO synthesis could play a role in the regulation of the inflammatory CNS damage and brain cancer progression. Recently, our laboratory has also found, in human astrocytic brain tumors, the presence of NPP1 protein, which is correlated with the tumor gradation (Aerts et al., 2011b).

In this communication, we studied the effect of activation of the NO/cGMP-pathway on the amount of NPP1 in C6 cells. Depending on the extracellular stimuli, cGMP is generated from GTP by either the membrane-bound/particulate (pGC) form or the cytosolic/soluble (sGC) form of guanylyl cyclase (reviewed in Feil and Kemp-Harper, 2006). Membrane-bound GC, activated by natriuretic peptides ANP, BNP or CNP, causes elevation of intracellular cGMP. Sorci et al. (1995) observed that all natriuretic peptides were activators of pGC in C6 cells

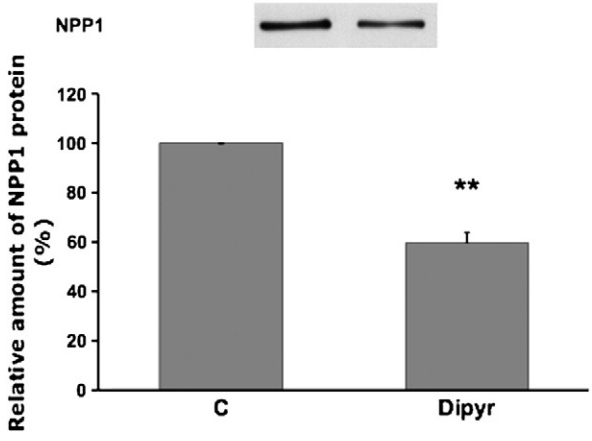

Fig. 5. Effect of cGMP-PDE inhibitor dipyridamole on the expression of NPP1. Cells were grown in chemically defined medium and were incubated with PDE inhibitor dipyridamole (Dipyr, 10 μM). Non-stimulated cells were incubated with PBS as control (C). After 48 h incubation, cells were harvested and analyzed for NPP1 protein by immunoblotting. Results are expressed as the relative amount of NPP1. The non-stimulated control cells were taken as 100%. The results are the mean ± S.E.M. of at least three independent experiments. The statistically significant differences in relative amount of NPP1 protein between non-stimulated and stimulated cells are indicated (** $P<0.01$).

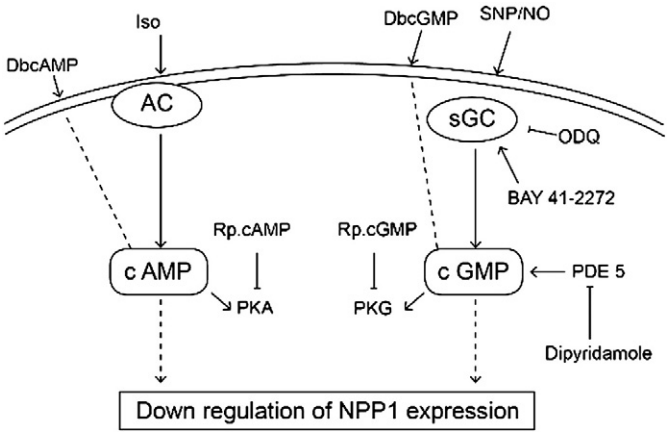

Fig. 6. Model summarizing the actions of the used inhibitors on the expression of NPP1. Cyclic AMP and cGMP initiate a cascade that down regulates the expression of NPP1. The use of the NO donor SNP and dbcGMP confirms the cGMP-dependent inhibition of NPP1 expression. Dipyridamole inhibits cGMP-specific PDE 5, thereby increasing the intracellular concentration of cGMP and inhibition of NPP1. (PKA, protein kinase A; AC, adenylate cyclase; Iso, (−)-isoproterenol; DbcAMP, N^6-O'^2-dibutyryl cAMP; DbcGMP, N^2-O'^2-dibutyryl cGMP; sGC, soluble guanylyl cyclase; PKG, protein kinase G; PDE 5, phosphodiesterase 5; NO, nitric oxide; SNP, sodium nitroprusside).

resulting in an increase in cGMP. In addition, Eguchi et al. (1992) found that CNP was more effective than ANP and BNP in these cells. Also Thiriet et al. (1997) found that CNP stimulates cGMP formation and gene induction of c-fos, TIS8/egr-1 and JunB, more potently than ANP or BNP. Although dbcGMP, which is intracellularly hydrolyzed to cGMP, decreases the amount of NPP1, we observed that CNP-induced cGMP formation had no effect on NPP1. We presume that the divergent effects of CNP and dbcGMP on the production of cGMP and on the amount of NPP1 are based on a difference in cGMP level. The level of cGMP induced by CNP is probably too small to achieve down regulation of NPP1.

Soluble GC is mainly present in cells of the central nervous system (Domek-Łopacińska and Strosznajder, 2005). NO, which is a direct activator of sGC and which is produced by NOS (eNOS, iNOS or nNOS), stimulates the synthesis of cGMP (Hanafy et al., 2001; Beavo and Brunton, 2002; Domek-Łopacińska and Strosznajder, 2005; Feil and Kemp-Harper, 2006). In C6 cells, we observed that NO-releasing agents (SNP) and membrane-permeable cGMP analogs (dbcGMP), but also cAMP inducing agents (dbcAMP and (−)-isoproterenol), inhibit the expression of NPP1. SNP, a disodium pentacyanonitrosylferrate(2-) dihydrate, down regulates NPP1 in C6 cells in a dose-dependent manner with a maximal effect in the range of 100–250 μM. It is well known that SNP forms a coordination complex of a ferrous ion (Fe^{2+}) with five CN^- anions and a nitrosonium ion (NO^+). It has been demonstrated that iron released by SNP in an ionic form plays a role in the suppression of NO production in C6 cells (Amoroso et al., 2000). So high SNP concentrations exert a protective effect on these cells due to the release of iron ions (Amoroso et al., 2000; Harhaji et al., 2004; Huitema et al., 2006). In addition, SNP increases $[Ca^{2+}]_i$ in C6 cells by enhancing the activity of the Na^+–Ca^{2+} exchanger of the NCX1 subtype (Amoroso et al., 1997, 2000; Annunziato et al., 2004). DbcAMP also causes an up regulation of the high molecular mass isoform of the exchanger, resulting in significant elevation of Na^+/Ca^{2+} exchanger activity (Xie et al., 2004). The two Ca^{2+}-dependent isoforms of NOS, nNOS and eNOS, are constitutively expressed in neuronal and astroglial cells (Sardón et al., 2004). However, BAPTA-AM, a cell-permeable intracellular Ca^{2+}-chelator, did not change the amount of NPP1, indicating that Ca^{2+} elevation is not involved in the regulation of NPP1 expression.

Because NO-releasing agents show similar effects as cGMP analogs on the level of NPP1, it is not unlikely that the effect is mediated by stimulation of the sGC activity. Although we observed no effect on the amount of NPP1 protein after stimulation with 5 μM BAY 41-2272, a NO-independent activator of sGC, a decrease of NPP1 protein was observed

when BAY 41-2272 was combined with SNP. In addition, our data showed a significant recovery of NPP1 protein amount if cells incubated with SNP were pre-treated with ODQ, an inhibitor of sGC. We may conclude that a NO-mediated activation of sGC causes a decrease of NPP1 protein by an elevated intracellular concentration of cGMP as illustrated in Fig. 6. Also dipyridamole, an inhibitor of cGMP-specific PDE5, down regulates NPP1.

We investigated a possible involvement of PKG in the down regulation of NPP1 by use of a specific inhibitor of PKG. C6 cells were pre-incubated with Rp-8*p*CPT-cGMPS (Rp-cGMP), a cell-permeable potent inhibitor of PKG. Besides the slight increase of NPP1 in comparison with control cells, no recovery of the level of NPP1 could be observed if cells were co-stimulated with Rp-cGMP and SNP. As is the case with PKA, PKG may also have a positive effect on the expression of NPP1. Ferrero et al. (2000) found that several PKG inhibitors increase the SNP-induced cGMP concentration. This could explain why NPP1 level in SNP stimulated cells pre-incubated with Rp-cGMP remains the same although an increase is observed after Rp-cGMP treatment in the absence of SNP stimulation. Our data do not indicate that PKG is involved in the cGMP-dependent down regulation of NPP1.

Recent studies have shown that besides PKG, high concentrations of cGMP can also cross-activate PKA, although with a 50-fold lower selectivity than cAMP (Cornwell et al., 1994; Hanafy et al., 2001; Pilz and Casteel 2003). A study of Zhang and Hintze (2001) suggests that the cAMP-dependent NO increase does not involve an increase in NOS activity. They suggest that cAMP increases NO through activation of PKA resulting in a downstream phosphorylation of eNOS by PKB through a PI 3-K-mediated effect (Zhang and Hintze 2001; Zhang and Hintze 2006). Our results indicate that cAMP-dependent inhibition of NPP1 by (−)-isoproterenol stimulation was not affected by inhibition of NOS with L-NAME. Furthermore, it has been shown that PKA and PKB are not involved in the cAMP-dependent inhibition of NPP1 expression (Aerts et al., 2011a).

In addition, use of Rp-cAMP, a selective potent inhibitor of PKA, also showed no significant difference in the amount of NPP1 as compared with untreated cells. After co-stimulation with 100 μM SNP, the amount of NPP1 recovers slightly when C6 cells were pre-incubated with Rp-cAMP. So, it is possible that PKA has a negative effect on the inhibitory action of cGMP, whereas cAMP-dependent down regulation of NPP1 works independently of PKA (Aerts et al., 2011a).

5. Conclusion

In summary, we have demonstrated that stimulation of C6 cells with dbcGMP or the NO-donor SNP causes a decrease in the amount of NPP1 protein. The inhibitory action of cGMP does not involve activation of PKG. Cyclic AMP and NO/cGMP activate two distinct pathways that do not converge at a common effector as shown by the use of specific inhibitors of both pathways.

Further research is needed to identify the cAMP and cGMP-dependent cascades involved in the inhibition of NPP1 expression in C6 cells. The elucidation of the mechanisms that regulate the expression of NPP1 could lead to new therapies or the designs of drugs that normalize its expression in pathologies where an aberrant expression is observed.

Acknowledgment

We thank Dr Hideyuki Sakoda of the Institute for Adult Disease, Asahi Life Foundation of Tokyo (Japan) for providing us the anti-NPP1 antibody. We thank Dr. Peter Williams of Janssen Research and Development for correcting the grammar and spelling of the manuscript. This work was supported by grants from BOF-NOI (HS). I. Aerts was a Research Assistant at the University of Antwerp.

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