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Expression of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in mouse tissues and cell lines using an antibody against the enzyme amino-terminal domain

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Abstract

We have produced a polyclonal antibody that specifically recognizes cGMP-binding cGMP-specific phosphodiesterase (PDE5). The antibody was raised in rabbit using as immunogen a fusion protein, in which glutathione *S*-transferase was coupled to a 171 amino acid polypeptide of the N-terminal region of bovine PDE5. The antibody is able to immunoprecipitate PDE5 activity from mouse tissues and neuroblastoma extracts while it has no effect on all other PDE isoforms present in the extracts. PDE5 activity recovered in the immunoprecipitates retains its sensitivity to specific inhibitors such as zaprinast (IC_{50} = 0.6 μM) and sildenafil (IC_{50} = 3.5 nM). Bands of the expected molecular mass were revealed when solubilized immunoprecipitates were analysed in Western blots. The antibody selectively stained cerebellar Purkinje neurones, which are known to express high levels of PDE5 mRNA. Western blot analysis of mouse tissues revealed the highest expression signal in mouse lung, followed by heart and cerebellum, while a lower signal was evident in brain, kidney and a very low signal was present in the liver. In the hybrid neuroblastoma-glioma NG108-15 cells the antibody revealed a high PDE5 induction after dibutyryl-cAMP treatment. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cyclic nucleotide phosphodiesterases (PDE) are a complex and expanding group of enzymes displaying different substrate specificities, kinetic and regulatory properties and tissue-specific distributions [1–4]. Recently PDE11 has been described as the last member of the known PDE family [5–7]. Whether each isoenzyme plays a specific role in different cell types and which role can be ascribed to specific PDE isoforms is still unclear, with a few exceptions, such as PDE6 in light transduction in the retina [8] and PDE3 in insulin action and in the regulation of insulin secretion [9].

PDE5 is a cGMP-specific isoform, initially discov-

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ered in bovine lung and rat platelets [10,11] and later found in several other tissues. Among these it has been described in vascular smooth muscle cells, where it plays a role in vascular relaxation mediated by the NO/cGMP pathway [12,13]. This finding has led to the suggestion that it may be involved in male erectile dysfunction and the PDE5 inhibitor sildenafil has recently been used in the treatment of this human disorder [14,15]. PDE5 has also been detected in the superior cervical ganglion [16] and in the brain, where its distribution appears to be confined to specific cellular components such as cerebellar Purkinje cells [17,18]. This restricted distribution suggests a possible role of the isoenzyme in specific neuronal populations. Although little is known about its specific role in neuronal function and differentiation, it should be recalled that PDE5 has been related to long-term depression (LTD) mediated by the NO/cGMP pathway in Purkinje cells and striatal spiny neurones [19,20].

PDE5 is a homodimer of two approx. 90–100 kDa subunits, the monomer containing two allosteric cGMP-binding sites whose function is not yet clear [21,22]. Binding of cGMP to both allosteric sites causes a conformational change in the enzyme, resulting in the exposure of N-terminal protein kinase (PK) G and PKA phosphorylation sites; although early reports indicate that phosphorylation of the purified enzyme had no effect on its catalytic properties [23,24], induction of activity by PKA has been observed in partially purified preparation of guinea pig lung enzyme [25] and in cultured smooth muscle cells [13]. PKA-PKG activation was recently observed using recombinant bovine PDE5 enzyme [26]. These data have raised questions concerning of the role of phosphorylation as a regulatory mechanism of PDE5 activity in intact cells. Two small putative PDE5 regulatory subunits of 14 and 18 kDa have recently been reported; they appear structurally related to the PDE6 inhibitory γ subunit, although their mechanism of action is still unclear [27].

We have previously shown that cGMP PDE activity in two neuronal cell lines, mouse neuroblastoma N18TG2 and the hybrid neuroblastoma-glioma NG108-15, is due to the presence of PDE5; we have also shown that PDE5 activity increases following treatment of neuroblastoma cells with dibutyryl (db)-cAMP [28]. Although a similar increase in enzyme activity has been observed in the two cell lines studied (N18TG2 and NG108-15), enhanced gene expression was observed only in the hybrid NG108-15 cells, while no increase in PDE5 mRNA was found in N18TG2 cells after dbcAMP treatment [29]. The complexity of PDE5 activity regulation, outlined above, underlies the need for a specific antibody probe to evaluate the quantitative changes in the expression of this enzyme at the protein level. Here we describe the production and characterization of a specific antibody raised against an N-terminal PDE5 region which was used to analyse the distribution of the enzyme in mouse tissues and its regulation in neuroblastoma clones during differentiation.

2. Materials and methods

2.1. Cell cultures

N18TG2, NG108-15 neuroblastoma cells and C6 glioblastoma cells were cultured as previously described [28]. 5×10^5 cells were plated on 90 mm culture dishes (Falcon). Twenty-four hours after plating, the medium was replaced with DMEM supplemented with either 10% FCS (control) or, for treated cells, with 1% FCS and 5 mM dbcAMP (Sigma, St. Louis, MO, USA) and changed after an additional 72 h. Five days after dbcAMP addition, both control and treated cells were washed twice with PBS and stored at −80°C.

2.2. Enzyme extracts

Cells were collected by scraping at 4°C in 20 mM Tris-HCl buffer pH 7.2, containing 0.2 mM EGTA, 5 mM $MgCl_2$, 1 mM PMSF, 5 mM β-mercaptoethanol, 10 μg/ml leupeptin, 5 μg/ml bestatin, 10 μg/ml pepstatin A, and homogenized with 20 strokes in a glass-teflon homogenizer. Homogenates were centrifuged at $100\,000\times g$ for 45 min at 4°C, the pellets were resuspended in homogenization buffer and centrifuged at $100\,000\times g$ for 45 min; the first and second supernatants were pooled. 0.1% Triton X-100 was included in the homogenization buffer in order to ensure the recovery in the supernatants of mem-

brane-associated PDE activity [28]. Tissue extracts from bovine lung and from adult Swiss CD1 mice were prepared following the procedure described for cell extracts. Protein concentration was determined as described by Bradford [30], using bovine serum albumin (BSA) as standard.

2.3. Anion-exchange chromatography and FPLC gel filtration

The enzyme extracts from each cell line were loaded onto a Mono Q anion-exchange column (HR 5/5, Pharmacia, Gaithersburg, MD, USA) in an FPLC system (Pharmacia). The flow rate was adjusted to 0.5 ml/min and the absorbance of the eluate monitored at 280 nm. Before loading the column was washed with buffer A (50 mM sodium acetate, pH 6.5, containing 0.2 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 5 mM NaF, 0.05% Triton X-100), and washing with the same buffer was continued for 10 min after loading the cell extract. Elution was then performed with a linear gradient of sodium acetate (0.05–1 M) in buffer B (sodium acetate, pH 6.5, containing 0.2 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 5 mM NaF, 0.05% Triton X-100) over 20 min and 45 fractions of 0.5 ml each were collected and assayed for PDE activity. PDE activity recovered in the eluate ranged from 85 to 100%. Fractions corresponding to the peak of PDE5 activity [28] were pooled and concentrated in a Centricon 10 (Amicon, Danvers, MA, USA) in a refrigerated centrifuge. This sample was loaded onto an FPLC Superose 12 HR 10/30 column (Pharmacia) and eluted at a flow rate of 0.25 ml/min with 20 mM HEPES buffer, pH 7.0 (containing 1 mM EGTA, 5 mM β-mercaptoethanol, 5 mM $MgCl_2$, 100 mM NaCl, 2 mM PMSF, 0.05% Triton X-100). Fractions with PDE activity were pooled, concentrated as described above and used for further analysis.

2.4. PDE isoforms preparation

Partially purified rat brain calcium-calmodulin stimulated phosphodiesterase (PDE1) was obtained in three chromatographic steps (DEAE-cellulose followed by CaM agarose affinity column and gel filtration) as already described [31]. This procedure gives an enzyme preparation showing a 6-fold stimulation of cGMP hydrolytic activity in the presence of 0.1 mM calcium and 3 μg/ml calmodulin. A partial purification of rat liver PDE2 activity was obtained in three chromatographic steps (DEAE-cellulose followed by ammonium sulphate precipitation and gel filtration) according to Yamamoto et al. [32]; this preparation showed a 5-fold stimulation of cAMP hydrolytic activity in the presence of 1 μM cGMP. A partial purification of PDE6 was obtained by anion exchange chromatography and gel filtration according to Baehr et al. [33].

2.5. PDE assay

PDE activity was determined following the two-step method described by Thompson and Appleman [34], in a final volume of 0.3 ml of assay buffer (60 mM HEPES pH 7.2, 0.1 mM EGTA, 5 mM $MgCl_2$, 0.5 mg/ml BSA, 30 μg/ml soybean trypsin inhibitor) using [^{3}H]cyclic GMP (specific activity 16.8 Ci/mmol) or [^{3}H]cyclic AMP (specific activity 28 Ci/mmol) (Amersham, Buckinghamshire, UK) as a substrate at a final concentration of 1 μM.

2.6. Production and purification of a PDE5-glutathione S-transferase fusion protein for immunization

A specific PDE5 antiserum was produced using as immunogen a fusion protein containing glutathione *S*-transferase (GST) and a 171 amino acid peptide corresponding to the 68–239 amino acid residues of the N-terminal bovine lung PDE5 sequence [35]. The fusion protein was obtained as follows.

RT-PCR amplification was performed on bovine lung poly(A)$^+$RNA (0.3 μg). Thirty-five PCR cycles were performed on cDNA after an initial denaturing step of 10 min at 91°C using the following profile: 91°C for 1 min; 52°C for 1 min; 72°C for 2 min using an oligonucleotide sense (5′-AGG GAT CCA ATC CTG CTC TTG CCC CTT GCAG-3′; nucleotides (nt) 298–320, with *Bam*HI site added on 5′) and antisense primer (5′-GAG GAT CCT CAT AGG CGT CTT TGA TGT TC-3′; nt 788–816, with *Bam*HI site included). The 517 nucleotide fragment obtained was then inserted into pGEX-3X vector

(Pharmacia Biotech) in frame with the GST gene present in the vector and cloned into DH5α bacteria [36,29]. One of the clones transfected with pGEX-3X containing the construct was stimulated for 3 h with 1 mM IPTG and the bacterial proteins were extracted by sonication of bacteria pellets resuspended in PBS (containing 1% Triton X-100, antipain 2 μg/ml, pepstatin 2 μg/ml, aprotinin 10 μg/ml, leupeptin 20 μg/ml). The supernatant obtained after 10 min centrifugation at 12 000×*g* at 4°C was filtered (0.45 μm) and loaded onto a glutathione-agarose column (Pharmacia Biotech), previously washed with PBS and equilibrated with PBS+1% Triton X-100 (4°C). After a wash with PBS, the GST-PDE5 fusion protein was eluted with 5 mM glutathione (Sigma) in 50 mM Tris-HCl, pH 8. Fractions obtained from the affinity chromatography were analysed by SDS-PAGE to verify the presence of the purified GST-PDE5 protein at the predicted molecular mass position (45.3 kDa). The purified product was then injected into a rabbit, following standard immunization procedures. Antiserum was collected and an anti-PDE5 antibody IgG fraction was purified by affinity chromatography on a protein A column (Amersham Pharmacia Biotech). The IgG fraction was eluted with 0.1 M glycine-HCl buffer, pH 3.0. The eluate was neutralized immediately with 1 M Tris-HCl, pH 8, and stored at −20°C until use. Pure GST peptide (26 kDa) was obtained by cloning pGEX-3X into DH5α bacteria and using the same 'in vitro' expression and purification procedure described for the fusion protein.

2.7. Immunoprecipitation

Sepharose CL-4B-protein-A resin (Pharmacia) was washed twice with Tris buffer saline (TBS) containing 0.1% BSA; 50 μl aliquots were incubated with increasing concentrations (2.4 ng–0.9 mg/ml) of PDE5 IgG fraction in a final volume of 0.3 ml. After 1–2 h at 25°C, the unbound antibody was removed by two washes in TBS/0.1% BSA. Aliquots of N18TG2 cells and mouse tissue were added and the mix (final volume of 0.5 ml) incubated for 2 h at 25°C in a rotating shaker. Following centrifugation of the mix, pellets containing immunoadsorbed proteins were first washed and then resuspended in TBS. Both supernatant and resuspended pellets were assayed for PDE activity in the absence and in the presence of PDE5-specific inhibitors. The same protocol using 0.9 mg/ml PDE5 IgG was used for immunoprecipitation of a mix of mouse tissue extracts. Control experiments were performed by replacing PDE IgG fraction with a non-immune IgG rabbit fraction (data not shown): in this case no PDE activity was detected in the pellet.

2.8. Western blot analysis

All samples analysed in Western blot were denatured with SDS-PAGE sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.0025% bromophenol blue), boiled and subjected to electrophoresis on an 8% SDS-polyacrylamide gel (SDS-PAGE) and then blotted onto nitrocellulose membranes (Protran, Schleicher& Schuell). Western blot analysis was performed by incubating the blots for 3 h with the anti-PDE5 antibody at a 1/2000 dilution; in some cases competition was performed by adding 10 μg/ml of GST-PDE5 or GST peptide together with the antibody. In one experiment an anti-GST antibody (Pharmacia Biotech) was used. A secondary anti-rabbit IgG alkaline phosphatase conjugated antibody was used to reveal the immunocomplexes and the bands stained with NBT (nitro blue tetrazolium) in the presence of BCIP (5-bromo-4-chloro-3-indolyl phosphate).

2.9. Immunocytochemistry

Two adult female mice (Strain CD1) were deeply anaesthetized with an i.p. injection of sodium pentobarbital (Pentothal Sodium, Farmaceutici Gellini, Aprilia, LT, Italy) and perfused transcardially with 200 ml of an oxygenated Ringer's solution, pH 7.3, followed by 500 ml of 4% (w/v) freshly made depolymerized paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4. The brains were dissected 1 h after termination of the perfusion and cryoprotected for 72 h in 30% (w/v) sucrose in saline, until they sank. Serial sagittal sections (25 μm thick) were cut on a cryostat and collected free floating in 0.5 M Tris-HCl, pH 7.6. The sections were incubated with 10% (v/v) methanol and 3% (v/v) H_2O_2 in 0.5 M Tris-HCl to inactivate endogenous peroxidases,

rinsed thoroughly in buffer, and blocked for 1 h in 5% (w/v) dry defatted milk and 0.5% Triton X-100 in Tris-HCl. The slices were incubated for 36 h at 4°C with the PDE5 antibody, diluted 1/400 in 0.5 M Tris-HCl containing 1% (w/v) dry milk and 0.2% Triton X-100. Following several rinses with buffer, the sections were incubated for 1 h at room temperature (RT) with 1:300 goat anti-rabbit IgG (Sternberger Monoclonal, Baltimore, MD, USA), rinsed again and incubated for 1 h at RT in 1:1000 rabbit peroxidase-anti-peroxidase (Sternberger Monoclonal). Finally, the sections were rinsed in Tris-HCl and antibody-binding sites were revealed by incubating with 0.05% DAB (3,3′-diaminobenzidine tetrahydrochloride, Sigma) and 0.01% H_2O_2 in Tris-HCl. Following thorough rinsing in buffer, the sections were dry mounted with cresyl gel (0.1% w/v gelatin in 80% v/v ethyl alcohol) and coverslipped with Eukitt balsam. Control sections, obtained either by omitting the primary antibody or by incubating with the PDE5 antibody preadsorbed with 150 μg/ml of the GST-peptide overnight at 4°C, were free of the immunoreaction product.

3. Results

3.1. Production and characterization of a PDE5-specific antiserum

To obtain a PDE5-specific antiserum, a PDE5-GST fusion protein was produced, purified and used as an immunogen in rabbits as described in Section 2. The 171 amino acid PDE5 peptide used was selected in the amino-terminal region of the protein for its divergence from other PDEs. A sequence comparison between this peptide and all SwissProt database using the FASTA3.3 program gave a 91–94% identity only with cloned (human, dog, rat) PDE5 isoforms. Low and scattered identity was found for PDE6 (18–19% considering human, bovine and mouse isoforms) and PDE2 isoforms (20.9–21.5% considering bovine, rat and human isoforms).

The presence of specific PDE5 antibodies in the IgG fraction was investigated in an immunoprecipitation assay on neuroblastoma cell extracts. Fig. 1A shows that the antibody precipitates PDE5 activity present in N18TG2 extracts in a concentration-de-

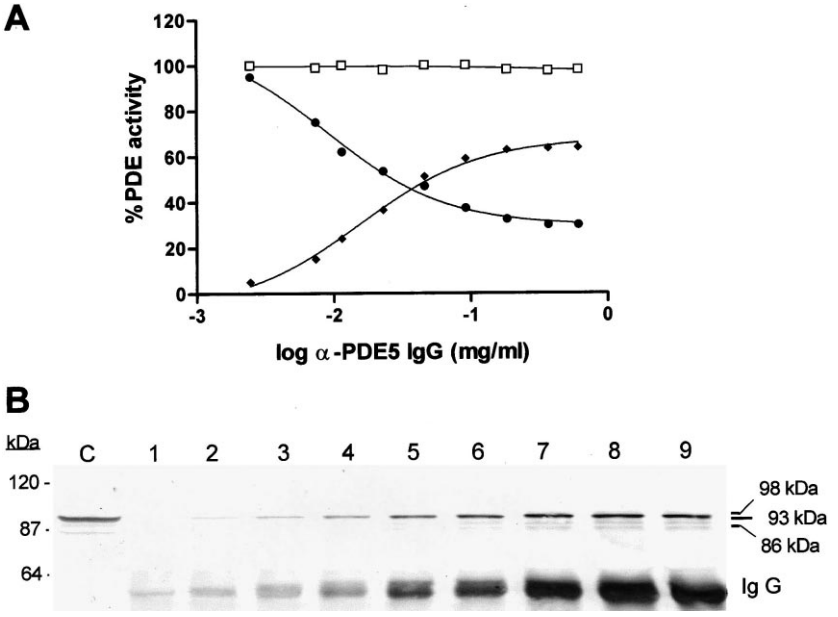

Fig. 1. Immunoprecipitation of PDE5 activity. Samples of N18TG2 cell extracts were incubated as described in Section 2 with increasing concentrations of PDE5 antibody (2.4, 7.4, 11.5, 23, 46, 92, 185, 370 and 600 ng/ml of IgG) (A) After centrifugation, cGMP PDE activity was measured in the supernatants (●) and in the pellets (♦). cAMP PDE activity in the same cell extracts was also measured in the supernatants (□). Calculated S.D. was in all cases $<5\%$ of the reported values. (B) The solubilized pellets were subjected to Western blot using the PDE5 antibody; lanes 1–9: from 2.4 to 600 ng/ml; in lane C supernatant of a N18TG2 cell extract loaded as control.

pendent manner. It also shows that the activity lost in the extracts subjected to immunoprecipitation is recovered in the pellet. On the other hand, cAMP PDE activity present in the N18TG2 extract, previously characterized as PDE4 [37], is not immunoprecipitated. When the immunoprecipitates were analysed by Western blot, one major band of 98 kDa and two minor bands of 93 and 86 kDa were revealed; their intensity is dependent on the concentration of the antibody (Fig. 1B). Inhibition studies were also performed on the immunoprecipitated N18TG2 PDE activity using the PDE5-specific inhibitors zaprinast and sildenafil (Fig. 2); the calculated IC_{50} values were distinctive of PDE5 isoforms (3.5 nM for sildenafil and 0.6 μM for zaprinast) [22]. To verify the specificity of antibody with respect to other PDEs we performed similar experiments using a mix of mouse tissue extracts containing the majority of PDE isoforms, including PDE2 (liver) and PDE6 (retina). The inhibition of PDE activity recovered in the resuspended pellet is reported in Fig. 2 and shows an IC_{50} (5 nM for sildenafil and 1 μM for zaprinast) very close to that obtained for N18TG2 immunoprecipitated activity. On the other hand, the Western blot analysis shown in Fig. 3 demonstrates a very low level of immunoreactivity for the PDE5 antibody and PDE1, PDE2 and PDE6 isoforms. The specificity of the antibody for PDE5

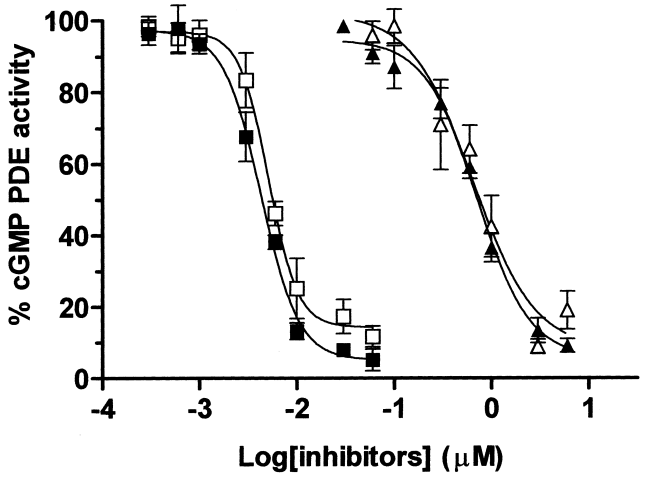

Fig. 2. Inhibition analysis of immunoprecipitated PDE5 activity. Inhibition by zaprinast (▲,△) and sildenafil (■,□) of PDE5 immunoprecipitated from N18TG2 cell (▲,■) and a mix of mouse lung, brain, kidney, retina, and liver extracts (△,□). PDE activity was assayed with 1 μM cGMP as substrate.

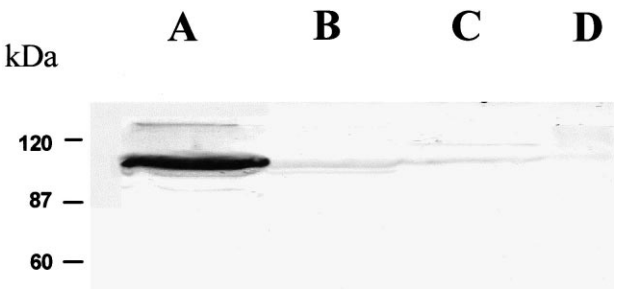

Fig. 3. Specificity of PDE5 antibody. Western blot analysis with PDE5 antibody; 50 μg of proteins were loaded in each lane. Lanes: A, N18TG2 cell extract used as control; B, rat liver PDE2; C, PDE6 from rat retina; D, rat brain PDE1. (For preparation see Section 2.)

is further confirmed by Western blot analysis of N18TG2 cell extracts subjected to successive steps of purification, as indicated in Section 2. Crude extracts and eluates from Mono Q anion-exchange chromatography and FPLC gel filtration were analysed (Fig. 4). When enriched preparations of PDE5 from N18TG2 and NG108-15 (lanes 1, 2 and 3) were loaded onto the gel, the staining intensity of immunoreactive bands paralleled the specific activity of the preparations (Table 1). It is interesting to note that, unlike the immunoprecipitates shown in Fig. 2, in N18TG2 crude and partially purified PDE5 preparations the intensity of the 98 and 86 kDa bands is almost the same while in the NG108-15 clone the two bands were not evident in the crude extract. In both neuroblastoma clones the 93 kDa band was not revealed. The specificity of the antiserum for PDE5 protein was finally shown by the lack of immunoreaction in C6 glioma cell extracts (Fig. 4) which only express PDE1 and PDE4 enzymes [38] and by peptide competition studies of Fig. 5 showing a Western blot analysis of N18TG2 in NG108-15 extracts in the presence and absence of peptide used as immunogen. This blot was overexposed to show unspecific low molecular mass bands. On the other hand, the antibody did not reveal any band in Western blot when GST protein alone was loaded onto the gel as shown in Fig. 6; GST and GST-PDE5 peptides are, however, strongly recognized by an anti-GST antibody. Lastly, the specificity of the antibody is further confirmed by immunocytochemistry of cerebellum sections showing that immunostaining is restricted to Purkinje cell bodies (Fig. 7A–C), as previously described in rat by in situ hybridization

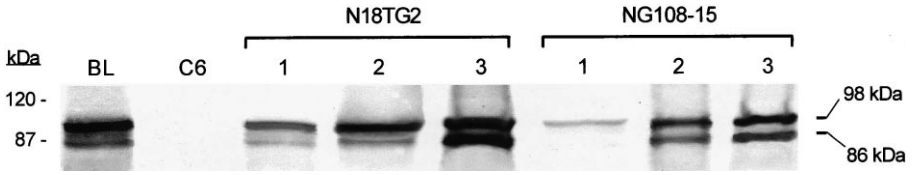

Fig. 4. PDE5 in neuroblastoma cells at successive purification steps. 20 μg of protein from N18TG2 and NG108-15 cell extracts and eluates of the indicated chromatographic columns were analysed by Western blot. Lanes: 1, cell extracts; 2, Mono Q anion-exchange chromatography; 3, FPLC gel filtration. 100 μg of bovine lung (BL) and rat glioma C6 (C6) were used respectively as positive and negative standards.

[39]. Immunostaining of Purkinje cells is abolished by omitting the primary antibody or by antibody preadsorbtion, as described in Section 2 (Fig. 7D).

3.2. PDE5 expression in mouse tissues

We used the antibody to analyse the distribution of PDE5 in several mouse tissues by Western blot. As shown in Fig. 8A, three immunoreactive bands (approx. 98, 93 and 86 kDa) were detected by the antibody as described above for neuroblastoma cell immunoprecipitates. A major band of about 98 kDa is highly expressed, mainly in mouse lung, but also in the cerebellum and heart; it shows a lower level of expression in the brain, excluding the cerebellum, and an intermediate level in the kidney, while it is not detectable in the liver. The other two bands show a weaker expression: in all cases the 93 kDa band is present in the lung and cerebellum, and a lower level can also be seen in the liver, brain and heart. The 86 kDa band is evident in the lung; it can be observed at lower levels in the heart, cerebellum, brain and kidney. The specificity of all three bands is demonstrated by their absence when GST-PDE5 peptide was added together with the antibody (Fig. 8B); on the other hand, the three bands persisted in the presence of pure GST, which was unable to compete with the antibody (not shown).

3.3. PDE5 expression during neuroblastoma cell differentiation

We used PDE5 antibody for Western blot analysis

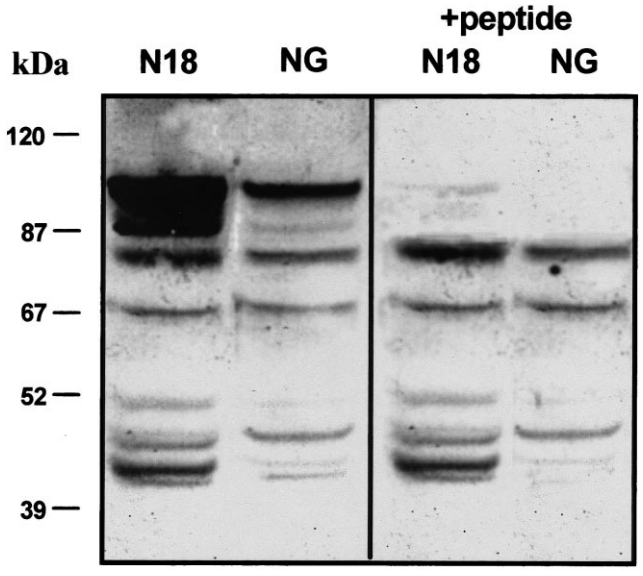

Fig. 5. Peptide competition study. Samples (50 μg of total protein) of N18TG2 and NG108-15 extracts were loaded in each lane and analysed by Western blot with PDE5 antibody in the presence and absence of 10 μg/ml of peptide. To reveal unspecific low molecular mass bands the blots are overstained with alkaline phosphatase detection solution.

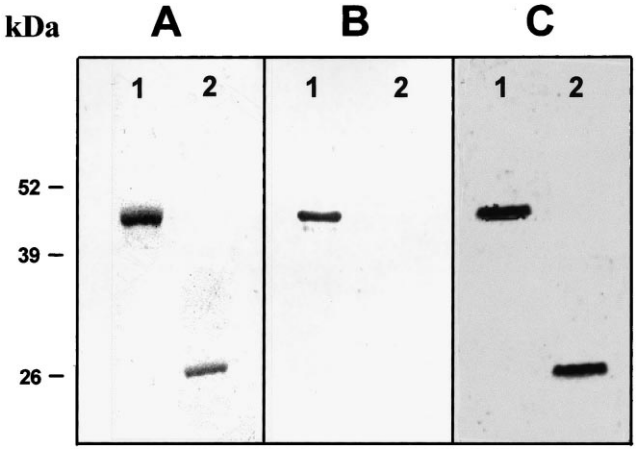

Fig. 6. PDE5 antibody specificities. SDS-PAGE of 10 μg of GST-PDE5 peptide (lane 1) and 6 μg of pure GST peptide (lane 2). (A) Coomassie blue stain; (B) Western blot using PDE5 antibody; (C) Western blot using an anti-GST antibody.

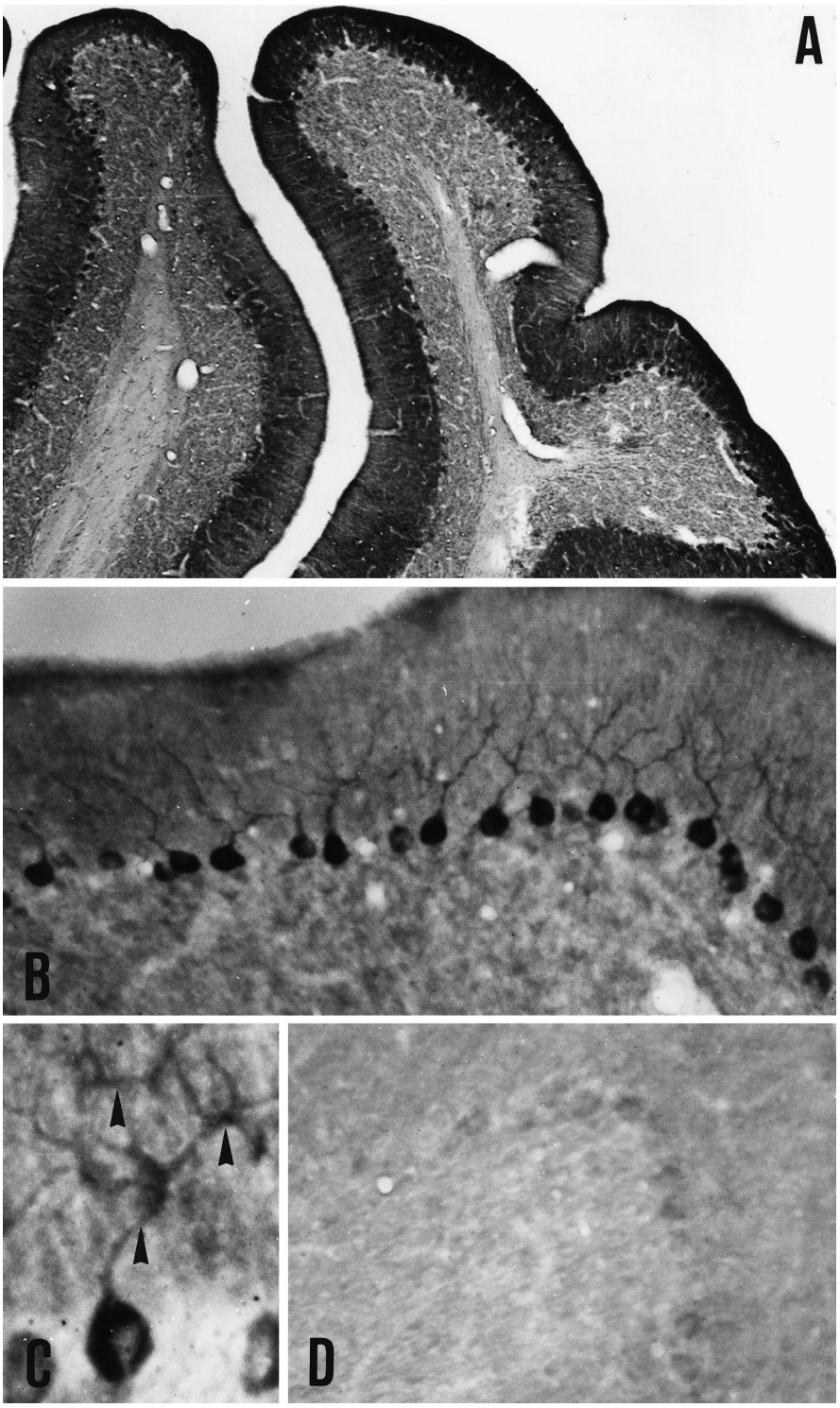

Fig. 7. PDE5 immunocytochemistry on sagittal sections of rat cerebellum. Immunoreactivity for PDE5 strongly labels Purkinje cell bodies (A–C) and dendrinites (B,C). Arrowheads in C point to the dendritic arborization of a Purkinje cell. (D) Control section obtained by preabsorption of the primary antibody with the GST–PDE5 peptide. A: ×60; B,D: ×240; C: ×620.

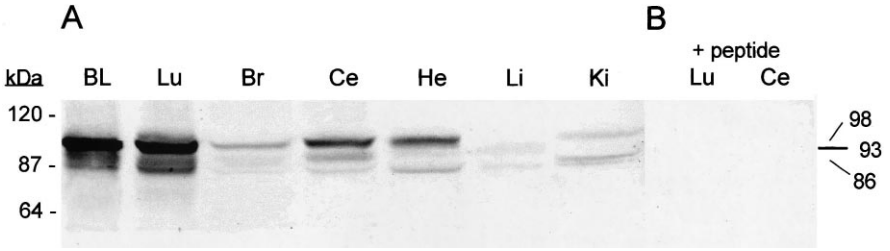

Fig. 8. PDE5 expression in mouse tissues. Western blot analysis was performed by loading in each lane 100 μg of protein from the indicated tissue extracts. On the right molecular masses (kDa) of the revealed bands are indicated. (A) Bovine lung (BL); mouse lung (Lu), brain (Br), cerebellum (Ce), heart (He), liver (Li), kidney (Ki). (B) Competition analysis using GST-PDE5 peptide (see Section 2) in mouse lung (Lu) and cerebellum extracts (Ce). The three specific bands are abolished by 10 μg/ml peptide.

of neuroblastoma clone extracts, to establish whether the level of the protein would confirm the data on the expression of PDE5 mRNA during differentiation [29]. The upper panel of Fig. 9 shows a representative Western blot analysis of N18TG2 and NG108-15 cell extracts under basal conditions and after 5 days of dbcAMP treatment. The level of the protein appears to be higher under differentiating conditions only in the hybrid clone, while in N18TG2 cells no significant change in the level of the protein can be observed. The lower panel of Fig. 9 (right side) shows densitometric analysis of the immunoreactive bands revealed in many different blots; the left side shows PDE5 activity assayed in the same experiments. While these data confirm that in NG108-15 cells the increase in PDE5 activity is related to an enhanced expression of the protein, they definitely show that, in N18TG2 cells, a higher level of PDE5 activity does not depend on a higher level of the enzymatic protein in the cells. Mechanisms other than upregulation of the protein expression must be operating in this case.

4. Discussion

In order to investigate PDE5 distribution in mouse tissue and the regulation of its expression in mouse neuroblastoma lines, we raised a polyclonal antiserum in rabbit, using as immunogen the N-terminal domain of the enzyme. Because of its low homology with other PDEs the N-terminal sequence seemed the most suitable to produce a PDE5-specific antiserum. This is because the peptide produced in the bacterial expression system corresponds to a unique regulatory region of the enzyme and overlaps with only a minor portion of one of the cGMP-binding tandem repeat sequences which are also found in PDE2 and PDE3 [35]. The specificity of the antibody obtained was demonstrated by several findings: (1) the decrease in cGMP hydrolytic activity in the supernatant of the N18TG2 extract in immunoprecipitation assays and the recovery of this activity in the immunoprecipitated pellets with inhibition properties specific to the PDE5 isoform; (2) the increase in PDE5 immunoreactive bands corresponding with the increase in PDE5-specific activity following N18TG2 chromato-

Table 1
Partial purification of PDE5 from neuroblastoma cell extracts

Purification step	N18TG2[a]			NG108-15[b]		
	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Purification (fold)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Purification (fold)
Cell extract	3.14	0.15		1.9	0.19	
Mono Q FPLC chromatography	1.8	0.36	2.3	0.31	0.64	3.4
FPLC gel filtration	0.82	0.61	3.86	0.16	1.44	7.6

PDE assays were performed at 1 μM cGMP as substrate. This purification scheme is typical of one performed at least three times.
[a]N18TG2 control cells.
[b]NG108-15 differentiated cells.

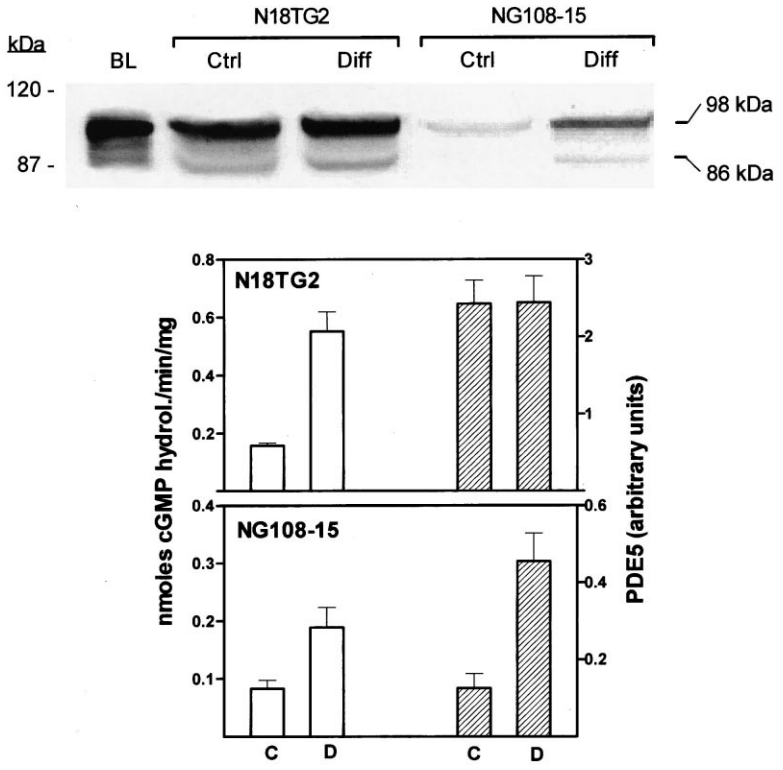

Fig. 9. Western blot analysis of PDE5 expression during neuroblastoma cell differentiation. (Upper panel) Representative Western blot of PDE5 bands revealed in dbcAMP treated (Diff) and control (Ctrl) neuroblastoma cell extracts using PDE5 antibody. 50 μg of protein were loaded in each lane. (Lower panel) Comparison in the control (C) and dbcAMP treated (D) neuroblastoma cell lines of cGMP-PDE activity and densitometric analysis of immunostained bands. PDE activity histograms are the mean of six observations (±S.E.). Histograms of immunostained bands represent the mean values (±S.E.) of five different experiments.

graphic purification steps; (3) the immunostaining of PDE5 in the cerebellum, showing strong and selective immunoreactivity in Purkinje cells, which coincides with the reported PDE5 mRNA localization [17]. Furthermore, using a mixture of mouse tissue extracts in which most PDE isoforms and splice variants are present, the antibody in question can immunoprecipitate cGMP-PDE activity with inhibition features specific to PDE5. Moreover, although a chimerically joined GST peptide was present in the peptide used as immunogen, our experiment produced no evidence of cross-reactivity with GST alone. Finally, the antibody showed a very low level of immunoreactivity with other PDEs. These finding prove that our antibody does not significantly recognize other phosphodiesterase isoforms besides PDE5.

The tissue distribution of PDE5 reported in Fig. 8 largely confirms data reported for human and rat tissue by Northern blot analysis [17,18,40,41] showing the highest expression in the lung; relatively high expression is also found in the cerebellum and heart and low expression in the liver and kidney. In addition to the 98 and 93 kDa bands, corresponding to the molecular masses of PDE5A1 and PDE5A2 respectively, a third band of approx. 86 kDa is revealed in all cases. This would appear to be a specific PDE5 signal, since it disappears when the antigen peptide is added to the antibody. At least two splice variants of mRNA, corresponding to PDE5A1 and PDE5A2, have been reported in human tissues [18]. The 86 kDa band may represent an additional splice variant, not yet described; alternatively, it may result from protein modification(s) undetectable in previous studies of mRNA distribution. The reported presence of a myristylation site in PDE5A1 [39] may be an example of such modifications.

With regard to the neuroblastoma lines we confirmed that the induction of PDE5 activity by cAMP occurs via two different mechanisms, as pre-

viously suggested by Northern blot analysis [28]. The constant intensity of the bands in N18TG2 cells after cAMP treatment indicates that in this case a more complex regulatory system is activated than in NG108-15. As noted, the phosphorylation state of the protein is dependent on cGMP binding to the allosteric enzyme site and this PKG-dependent phosphorylation causes an increase in catalytic activity [13,26]. On the other hand, in airway smooth muscle cells a PKA-dependent phosphorylation of PDE5 has been shown to be prevented by the PDE6γ inhibitory subunit. Two small proteins, immunologically related to PDE6γ, have been found associated with PDE5 in these cells and have been proposed as a possible regulatory mechanism for PDE5 [27]. A similar system may be responsible for the induction of PDE5 activity in N18TG2 cells, when they are exposed to dbcAMP treatment, with a consequent activation of PKA-mediated phosphorylation of the enzyme. Although further work is needed to clarify whether this regulation system is present in N18TG2, the data presented here clearly show that the two cell lines follow different pathways for the regulation of PDE5 activity.

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References

[1] J.A. Beavo, Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms, Physiol. Rev. 75 (1995) 725–748.

[2] F. Burns, A.Z. Zhao, J.A. Beavo, Cyclic nucleotide phosphodiesterases: gene complexity, regulation by phosphorylation, and physiological implications, Adv. Pharmacol. 36 (1996) 29–48.

[3] S.H. Soderling, J.A. Beavo, Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions, Curr. Opin. Cell Biol. 12 (2000) 174–179.

[4] S.H. Soderling, S.J. Bayuga, J.A. Beavo, Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A, Proc. Natl. Acad. Sci. USA 96 (1999) 7071–7076.

[5] L. Fawcett, R. Baxendale, P. Stacey, C. McGrouther, I. Harrow, S. Soderling, J. Hetman, J.A. Beavo, S.C. Phillips, Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A, Proc. Natl. Acad. Sci. USA 97 (2000) 3702–3707.

[6] J.M. Hetman, M. Robas, R. Baxendale, M. Fidock, S.C. Phillips, S.H. Soderling, J.A. Beavo, Cloning and characterization of two splice variants of human phosphodiesterase 11A, Proc. Natl. Acad. Sci. USA 97 (2000) 12891–12895.

[7] K. Yuasa, J. Kotera, K. Fujishige, H. Michibata, T. Sasaki, K. Omori, Isolation and characterization of two novel phosphodiesterase PDE11A variants showing unique structure and tissue-specific expression, J. Biol. Chem. 275 (2000) 31469–31479.

[8] I. Lagnado, D. Baylor, Signal flow in visual transduction, Neuron 8 (1992) 995–1002.

[9] V.C. Manganiello, E. Degerman, C.J. Smith, V. Vasta, H. Tornqvist, P. Belfrage, Mechanisms for activation of the rat adipocyte particulate cyclic-GMP-inhibited cyclic AMP phosphodiesterase and its importance in the antilipolytic action of insulin, Adv. Second Messenger Phosphoprotein Res. 25 (1992) 147–164.

[10] S.H. Francis, J.D. Corbin, Purification of cGMP-binding protein phosphodiesterase from rat lung, Methods Enzymol. 159 (1988) 722–729.

[11] P. Hamet, J. Tremblay, Platelet cGMP-binding phosphodiesterase, Methods Enzymol. 159 (1988) 710–722.

[12] S.H. Francis, M.K. Thomas, J.D. Corbin, Cyclic GMP-binding cyclic GMP-specific phosphodiesterase from lung, in: J.A. Beavo, M.D. Houslay (Eds.), Cyclic Nucleotide Phosphodiesterase: Structure, Regulation and Drug Action, John Wiley and Sons, Chichester, 1990, pp. 117–140.

[13] T.A. Wyatt, A.J. Naftilan, S.H. Francis, J.D. Corbin, ANF elicits phosphorylation of the cGMP phosphodiesterase in vascular smooth muscle cells, Am. J. Physiol. 274 (1998) H448–H455.

[14] M. Boolell, S. Gepi-Attee, J.C. Gingell, M.J. Allen, Sildenafil, a novel effective oral therapy for male erectile dysfunction, Br. J. Urol. 78 (1996) 257–261.

[15] J.Y. Jeremy, S.A. Ballard, A.M. Naylor, M.A.W. Miller, G.D. Angelini, Effect of sildenafil, a type-5 cGMP phosphodiesterase inhibitor, and papaverine on cyclic GMP and cyclic AMP levels in the rabbit corpus cavernosum in vitro, Br. J. Urol. 79 (1997) 958–963.

[16] M. Giorgi, R. Squitti, P. Bonsi, P. Paggi, G. Toschi, Activities of 3′:5′ cyclic nucleotide phosphodiesterases in the superior cervical ganglion of rat: characterization, compartmentalization and observations in young and old animals, Neurochem. Int. 25 (1994) 493–500.

[17] J. Kotera, N. Yanaka, K. Fujishige, Y. Imai, H. Akatsuka, T. Ishizuka, K. Kawashima, K. Omori, Expression of rat cGMP-binding cGMP-specific phosphodiesterase mRNA in

Purkinje cell layers during postnatal neuronal development, Eur. J. Biochem. 249 (1997) 434–442.
[18] K. Loughney, T.R. Hill, V.A. Florio, L. Uher, G.J. Rosman, S.L. Wolda, A.B. Jones, M.L. Howard, L.M. McAllister-Lucas, W.K. Sonnenburg, S.H. Francis, J.D. Corbin, J.A. Beavo, K. Ferguson, Isolation and characterization of cDNAs encoding PDE5A, a human cGMP-binding cGMP-specific 3′,5′-cyclic nucleotide phosphodiesterase, Gene 216 (1998) 139–147.
[19] N.A. Hartell, Inhibition of cGMP breakdown promotes the induction of cerebellar long-term depression, J. Neurosci. 16 (1996) 2881–2890.
[20] P. Calabresi, P. Gubellini, D. Centonze, G. Sancesario, M. Morello, M. Giorgi, A. Pisani, G. Bernardi, A critical role of the nitric oxide/cGMP pathway in corticostriatal long-term depression, J. Neurosci. 19 (1999) 2489–2499.
[21] M.K. Thomas, S.H. Francis, J.D. Corbin, Characterization of a purified bovine lung cGMP-binding cGMP phosphodiesterase, J. Biol. Chem. 265 (1990) 14964–14970.
[22] J.D. Corbin, S.H. Francis, Cyclic GMP phosphodiesterase-5: target of sildenafil, J. Biol. Chem. 274 (1999) 13729–13732.
[23] M.K. Thomas, S.H. Francis, J.D. Corbin, Substrate- and kinase-directed regulation of phosphorylation of a cGMP-binding phosphodiesterase by cGMP, J. Biol. Chem. 265 (1990) 14971–14978.
[24] I.V. Turko, S.H. Francis, J.D. Corbin, Binding of cGMP to both allosteric sites of cGMP-binding cGMP-specific phosphodiesterase (PDE) is required for its phosphorylation, Biochem. J. 329 (1998) 505–510.
[25] F. Burns, I.W. Rodger, N.J. Pyne, The catalytic subunit of protein kinase A triggers activation of the type V cyclic GMP-specific phosphodiesterase from guinea-pig lung, Biochem. J. 283 (1992) 487–491.
[26] J.D. Corbin, I.V. Turko, A. Beasley, S.H. Francis, Phosphorylation of phosphodiesterase-5 by cyclic nucleotide-dependent protein kinase alters its catalytic and allosteric cGMP-binding activities, Eur. J. Biochem. 267 (2000) 2760–2767.
[27] A. Lochhead, E. Nekrasova, Y. Arshavsky, N.J. Pyne, The regulation of the cGMP-binding cGMP phosphodiesterase by proteins that are immunologically related to gamma subunit of the gamma subunit of the photoreceptor cGMP phosphodiesterase, J. Biol. Chem. 272 (1997) 18397–18403.
[28] M. Giorgi, D. Giordano, C. Caniglia, S. Biagioni, G. Augusti-Tocco, Induction of cyclic AMP and cyclic GMP 3′:5′-cyclic nucleotide phosphodiesterase activities in neuroblastoma lines under differentiating conditions, Int. J. Dev. Neurosci. 15 (1997) 309–319.
[29] D. Giordano, M. Giorgi, C. Sette, S. Biagioni, G. Augusti-Tocco, cAMP-dependent induction of PDE5 expression in murine neuroblastoma cell differentiation, FEBS Lett. 446 (1999) 218–222.
[30] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein, Anal. Biochem. 72 (1976) 248–254.
[31] R.L. Kincaid, V.C. Manganiello, C.E. Odya, J.C. Osborne, I.E. Stith-Coleman, M.A. Danello, M. Vaughan, Purification and properties of calmodulin-stimulated phosphodiesterase from mammalian brain, J. Biol. Chem. 259 (1984) 5158–5166.
[32] T. Yamamoto, V.C. Manganiello, M. Vaughan, Purification and characterization of cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from calf liver, J. Biol. Chem. 258 (1983) 12526–12533.
[33] W. Baehr, M.J. Devlin, M.L. Applebury, Isolation and characterization of cGMP phosphodiesterase from bovine rod outer segments, J. Biol. Chem. 254 (1979) 11669–11677.
[34] W.J. Thompson, M.M. Appleman, Multiple cyclic nucleotide phosphodiesterase activities from rat brain, Biochemistry 10 (1971) 311–316.
[35] L. McAllister-Lucas, W.K. Sonnenburg, A. Kadlecek, D. Seger, H. Le Trong, J.L. Colbran, M.K. Thomas, K.A. Walsh, S.H. Francis, J.D. Corbin, J.A. Beavo, The structure of a bovine lung cGMP-binding, cGMP-specific phosphodiesterase deduced from a cDNA clone, J. Biol. Chem. 268 (1993) 22863–22873.
[36] F.M. Ausubel, R. Brent, R.E. Kingstone, D.D. Moore, J.G. Seidman, J.A. Smith, L. Struhl, Current Protocols in Molecular Biology, Wiley, New York, 1987–1997.
[37] M. Giorgi, C. Caniglia, G. Scarsella, G. Augusti-Tocco, Characterization of 3′:5′ cyclic nucleotide phosphodiesterase activities of mouse neuroblastoma N18TG2 cells, FEBS Lett. 324 (1993) 76–80.
[38] P. Onali, J.P. Schwartz, I. Hanbauer, E. Costa, Regulation by a β-adrenergic receptor of a Ca^{2+}-independent adenosine 3′,5′-(cyclic)monophosphate phosphodiesterase in C6 glioma cells, Biochim. Biophys. Acta 675 (1981) 285–292.
[39] J. Kotera, K. Fujishige, H. Akatsuka, Y. Imai, N. Yanaka, K. Omori, Novel alternative splice variants of cGMP-binding cGMP-specific phosphodiesterase, J. Cell Biol. 273 (1998) 26982–26990.
[40] P. Stacey, S. Rulten, A. Dapling, S.C. Phillips, Molecular cloning and expression of human cGMP-binding cGMP-specific phosphodiesterase (PDE5), Biochem. Biophys. Res. Commun. 247 (1998) 249–254.
[41] N. Yanaka, J. Kotera, A. Ohtsuka, H. Akatsuka, Y. Imai, H. Michibata, K. Fujishige, E. Kawai, S.I. Takebayashi, K. Okumura, K. Omori, Expression, structure and chromosomal localization of the human cGMP-binding cGMP-specific phosphodiesterase PDE5A gene, Eur. J. Biochem. 255 (1998) 391–399.