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cAMP-dependent induction of PDE5 expression in murine neuroblastoma cell differentiation

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Abstract The present study demonstrates, in both hybrid NG108-15 and mouse neuroblastoma N18TG2 cells, the presence and regulation of PDE5 mRNA during cell differentiation. PDE5 cDNA probes in Northern blot analysis recognize a ~9 kb transcript in bovine lung as well as in mouse neuroblastoma cells. Hybridization on total RNA extracted from dibutyryl-cAMP-treated NG108-15 cells shows a 5-fold increase of PDE5 9 kb mRNA: such an increase is not observed in N18TG2 although we observed a similar increase in the enzymatic activity of both cell lines. Our data demonstrate that PDE5 gene expression can be regulated by cAMP and suggest the existence of a complex regulatory system for PDE5 activity.

Key words: PDE5; cAMP-dependent expression; Differentiation; Neuroblastoma; NG108-15

1. Introduction

cAMP has been suggested to play a critical role in the nervous system development [1–11]. On the other hand, the signalling cascade activated by cAMP is also capable of interacting with the cGMP pathway [12,13].

Phosphodiesterases (PDEs) are recognized as important elements in the cyclic nucleotide signalling system [14–16]. In the nervous system, as in other tissues, more than one PDE isoenzymes are expressed [14]. Among them, little is known on PDE5 (cGMP-binding cGMP-specific) expression in the nervous system. It has been described in neuronal cell lines [17,18], in rat superior cervical ganglion [19] and in cerebellum Purkinje cells [20].

PDE5 is a homodimer of two ~100 kDa subunits, first purified and characterized from rat [21] and bovine lung [22]. PDE5 cloning data [20,23–26] confirm that the monomer contains two allosteric cGMP-binding sites, whose function is still unknown. In spite of the extensive characterization and distribution studies of PDE families, no clear correlation has been demonstrated between a given PDE isoenzyme and a specific neuronal function, with the exception of PDE6 in the visual system [27].

The major hindrance in establishing a functional role for PDE isoforms can be ascribed to the brain cellular and functional complexity. Mouse neuroblastoma lines have been widely used as model system of homogeneous cell populations. Two lines, N18TG2 and the derived NG108-15 (108CC15) neuroblastoma × glioma hybrid, appear particularly interesting for their different abilities to express neurospecific markers [28–32]. This had led to consider them as representative of two phases of neurone differentiation, respectively characterized by fiber formation and synaptogenesis.

We have previously shown that two PDE isoforms, PDE4 and PDE5, are expressed in both cell lines and their activity is induced by dbcAMP [18]. The cAMP level is known to regulate the transcription of many genes, among these transcriptional regulation of PDE4 activity has been reported as a feedback regulatory response to increased cellular cAMP level [33,34]. Although regulation of PDE5 activity by cAMP through PKA phosphorylation has been reported in the respiratory system [35,36], no evidence of transcriptional regulation of PDE5 by cAMP has been described so far. Thus it appeared of interest to establish whether cAMP may act as an activator of PDE5 transcription gene(s), as well as for PDE4.

To this aim, we produced PDE5-specific cDNA probes by reverse transcriptase polymerase chain reaction (RT-PCR). Here we report the results of Northern blot analysis of PDE5-mRNA levels in neuroblastoma cells maintained in basal and differentiating (dbcAMP treatment) conditions.

2. Materials and methods

2.1. Cell cultures

N18TG2 and NG108-15 cells were cultured as previously described [18]. 3.5×10^5 cells were plated on 90 mm culture dishes (Falcon). 24 h after plating, the medium was replaced with either DMEM supplemented with 10% FCS (control) or supplemented with 1% FCS and 5 mM dbcAMP (N^6,2′-*o*-dibutyryladenosine 3′:5′-cyclic monophosphate, Sigma Chemical) and changed after an additional 72 hrs. Five days after dbcAMP addition, both control and treated cells were washed twice with PBS and stored at −80°C.

2.2. PDE assay

Extraction of the PDE activity was performed as previously described [18]. Cells were homogenized at 4°C on 20 mM Tris-HCl buffer pH 7.2, containing 0.2 mM EGTA, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 mM β-mercaptoethanol, 10 μg/ml leupeptin, 5 μg/ml bestatin, 10 μg/ml pepstatin A, 0.1% Triton X-100, in a glass-teflon homogenizer with 20 strokes. Homogenates were centrifuged at 100 000×*g* for 45 min at 4°C, pellets were resuspended in homogenization buffer and centrifuged at 100 000×*g* for 45 min: the first and second supernatants were pooled. Triton was included in the homogenisation buffer in order to ensure the recovery of membrane-associated PDE activity [17]. PDE activity was determined with the two step method as described by Thompson and Appleman [37] 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]cAMP (specific activity 28 Ci/mmol) or [^{3}H]cGMP (specific activity 16.8 Ci/mmol) (Amersham) as a substrate at a final concentration of 1 μM.

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2.3. Isolation of total and poly-A$^+$ RNAs

Total RNA was prepared from bovine lung tissue and neuroblastoma cells using TRIzol reagent according to the manufacturer's instructions (Life Technologies). poly-A$^+$ RNA was purified from total RNA preparations using an oligo(dT) cellulose column according to the method described by Sambrook et al. [38].

2.4. RT-PCR

RT-PCR analysis was performed with the Stratagene RT-PCR kit using *Pfu* DNA polymerase or *Taq plus* DNA polymerase (Stratagene). The first strand cDNA was synthesized by reverse transcription of total RNA (2–6 μg) or poly-A$^+$ RNA (0.3 μg) from bovine lung and N18TG2 or NG108-15 total RNA (9 μg) using random hexamers. Controls, omitting reverse transcriptase, were included in the reactions. The following PCR sense and antisense primers designed on the bovine lung PDE5 cDNA clone [23] were used and conventionally denoted respectively MI (5′-GGT TTG ATA ATG ATG AAG GGG ACCA-3′: nucleotides 487–511) and MII (5′-CAT CAC GTT CCC GTG TTA AAG TATC-3′: nucleotides 1254–1278). The two oligonucleotides were paired to amplify a 792 bp product corresponding to the 130–393 amino acid residues of the N-terminal PDE5 bovine lung sequence. 35 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. The bovine lung, N18TG2 and NG108-15 amplified products obtained with the MI and MII primers were named respectively MPDE5BL, MPDE5N18 and MPDE5NG. In this notation the first letter identifies the primers used for amplification and the final letters the source of mRNA (BL for bovine lung, N18 and NG for the two neuroblastoma clones). RT-PCR analysis was performed also on mouse N18TG2 total RNA using two different sense and antisense oligonucleotide primers designed on bovine lung PDE5 sequence. They were conventionally denoted respectively as MCs (5′-AGG GAT CCA ATC CTG CTC TTG CCC CTT GCAG-3′: nucleotides 298–320, with a *Bam*HI site added on 5′) and MCa (5′-GAG GAT CCT CAT AGG CGT CTT TGA TGT TC-3′: nucleotides 788–816, with a *Bam*HI site included) (Biogen). These primers are more conserved with respect to the MI and MII primers in the bovine and rat lung sequence [23,20]. The resulting amplified 517 nucleotide fragment corresponds to the 68–239 amino acid residues of the N-terminal bovine lung PDE5 sequence. The N18TG2 product amplified with MCs and MCa primers was named MCPDE5N18. This amplification product was named following the criteria outlined above, with MC indicating the primers used in RT-PCR.

2.5. Cloning of the bovine lung and N18TG2 PDE5 cDNA fragments

The 792 bp PCR product MPDE5BL was inserted into the pCR-Script vector (pCR-Script Amp SK(+) Cloning Kit, Stratagene) and DH5α bacteria were transformed with the resulting vector construct. The bovine lung cDNA insert was characterized by restriction analysis (*Bam*HI, *Hin*dIII) and sequenced by the dideoxynucleotide sequencing method with Sequenase (United States Biochemical). The 517 bp PCR product MCPDE5N18 was inserted into the pGEX-3X vector (*Bam*HI site) and cloned using DH5α bacteria. The sequence homology of the mouse N18TG2 amplified product and bovine and rat lung clones was verified by restriction analysis in conserved restriction sites (*Hin*dIII, *Eco*RI).

2.6. Northern blot analysis

Both PDE5 cDNA from bovine lung (MPDE5BL) and the neuroblastoma clone (MCPDEN18) were used in Northern blot analysis. 25 μg of total RNA was fractionated on a formaldehyde/1.2% agarose gel and transferred onto a Hybond-N nylon membrane (Amersham) or an Immobilon-Ny+ nylon membrane (Millipore). Blots were prehybridized at 45°C for 3 h in QuiKHyb solution (Stratagene) and for 30 min in a solution containing 50% formamide, 6×SSPE pH 7.4, SX Denhardt's solution, 5% dextran sulfate, 0.5% SDS, 0.5 mg/ml yeast tRNA and 0.1 mg/ml of denaturated salmon sperm DNA. A random hexanucleotide primer-labelled ([^{32}P]dα-ATP) probe was prepared using both MPDE5BL, excized from pCR-Script by digestion with *Kpn*I and *Sac*I, and MCPDE5N18 excized from pGEX-3X using *Bam*HI enzyme. Hybridization was performed overnight at 45°C. Membranes were washed for 30 min with 2×SSPE, 0.1% SDS at room temperature and twice with 0.1×SSPE, 0.1% SDS at 50°C. Blots were subjected to autoradiography with BIOMAX-MS Kodak film for 32–48 h or X-OMAT Kodak film for 120–144 h. Densitometric analysis was performed using the Phoretix 1D program, which evaluates the band volume as the sum of the intensity of every pixel in the band. To normalize the RNA amount in the different lanes, blots, after probe stripping, were hybridized with a 1.2 kb GAPDH fragment probe. Six filters were hybridized under standard conditions for both probes and the ratio of intensity of PDE5/GAPDH bands was used to normalise the PDE5 band intensity. The standard error was calculated to evaluate analytical variations.

3. Results

NG108-15 neuroblastoma glioma hybrid cells respond to cAMP extending neurites, expressing high levels of neuro-specific proteins and establishing functional synapses [30,31,39]. When subjected to the same treatment, the parental neuroblastoma N18TG2 clone shows a similar morphological differentiation, even though this is not accompanied by the induction of neurotransmission apparatus components [40]. Chromatographic analysis, studies of kinetic properties and sensitivity to specific inhibitors demonstrated the presence in both clones of two PDE isoforms :PDE4 and PDE5. In both cell lines their activity is higher in the presence of dbcAMP [17,18]. The time course of PDE4 and PDE5 activity induction suggested a possible involvement of transcription in the mechanisms of enzyme regulation activated by cAMP [18]. We have focused our attention on the induction of PDE5 since only post-translational regulation by cAMP has so far been reported for this PDE isoform [35,36], while cAMP activation of PDE4 transcription is known [33,34].

We produced cDNA PDE5 probes by RT-PCR, using mRNA preparation from bovine lung (in which PDE5 has first been described and characterized). RT-PCR was then also performed with N18TG2 and NG108-15 neuroblastoma mRNA preparations. Both bovine and murine probes were used to reveal specific mRNA bands in undifferentiated and dbcAMP treated N18TG2 and NG108-15 cells. PDE assays were also performed on cultures run in parallel to those used for RNA extraction. They confirmed that, following dbcAMP treatment, both cell lines displayed a higher level of PDE5 (Table 1).

Table 1
Induction of PDE5-specific activities (nmoles cGMP hydrolysed/min/mg of protein) in N18TG2 and NG108-15 differentiated cells

Clones	PDE5 activity		activity increase
	Ctrl	dbcAMP	(fold)
N18TG2	0.157 ± 0.01	0.552 ± 0.05	3.5
NG108-15	0.083 ± 0.01	0.190 ± 0.03	2.3

Note: Ctrl = control cultures; dbcAMP = 5 days dbcAMP (5 mM) treated cultures.
Data are expressed as means ± S.E. (n = 6).

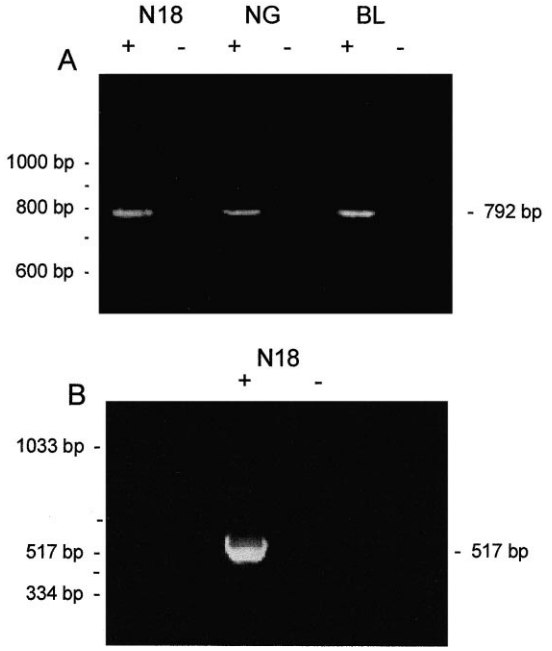

Fig. 1. RT-PCR using specific PDE5 primers performed on both bovine and neuroblastoma RNAs. A: RT-PCR using MI and MII PDE5-specific primers (see Section 2) amplified a 792 bp fragment from bovine lung RNAs (BL) as well as from neuroblastoma N18TG2 (N18) and NG108-15 (NG) RNAs. B: RT-PCR using MCs and MCa PDE5-specific primers (see Section 2) amplified a 517 bp fragment from neuroblastoma N18TG2 RNA. Amplifications were performed either with reverse transcriptase (+) or without (−) as control.

3.1. *RT-PCR production and cloning of a cDNA PDE5 probe from bovine lung and neuroblastoma*

MI and MII primers (see Section 2), designed on bovine lung sequence [23], were selected to amplify a region within a domain that shares a low level of homology with other PDE families. The 792 bp fragment comprised by MI and MII primers encodes 263 amino acid residues (130–393 in the bovine lung protein) of the PDE5 N-terminal region. It contains 12 amino acid residues of the unique PDE5 sequence and 251 amino acid residues of the cGMP-binding region [23]. These primers in RT-PCR, performed either on total (Fig. 1A) or poly-A$^+$ RNA from bovine lung, amplified the expected 792 bp fragment (MPDE5BL). The amplified fragment was ligated into the plasmid vector pCR-Script Amp SK(+) and cloned in DH5α. Restriction analysis with *Bam*HI or *Hin*dIII gave fragments of the expected size. Moreover, the sequence between nucleotide 549 and 710 of the amplified fragment showed a 100% identity with the bovine lung PDE5 cDNA.

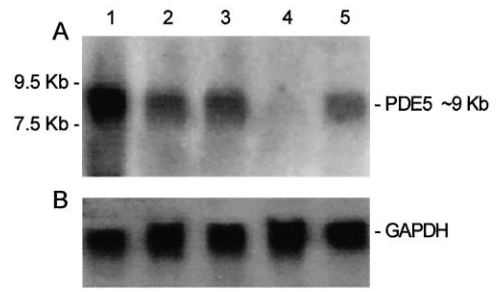

Fig. 2. Expression of PDE5 mRNA in dbcAMP treated neuroblastoma cells using the bovine lung probe (MPDE5BL). A: Northern blot analysis using ^{32}P-labelled bovine lung PDE5 cDNA probe (MPDE5BL, as described in Section 2). 25 μg of total RNAs from bovine lung (lane 1), control and 5 days dbcAMP-treated N18TG2 cells (lanes 2 and 3, respectively), control and dbcAMP-treated NG108-15 cells (lanes 4 and 5, respectively). Markers are indicated on the left of the panel. B: The same membrane was re-hybridized with a ^{32}P-labelled GAPDH probe to normalize the amount of RNA.

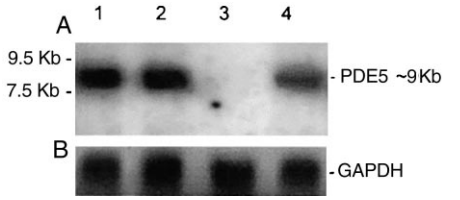

Fig. 3. Expression of PDE5 mRNA in dbcAMP-treated neuroblastoma cells using the murine neuroblastoma probe (MCPDE5N18). A: Northern blot analysis using ^{32}P-labelled mouse neuroblastoma PDE5 cDNA probe (MCPDE5N18, as described in Section 2). 25 μg of total RNAs from control and 5 days dbcAMP-treated N18TG2 cells (lane 1 and 2, respectively), control and dbcAMP-treated NG108-15 cells (lanes 3 and 4, respectively). Markers are indicated on the left of the panel. B: The same membrane was re-hybridized with a ^{32}P-labelled GAPDH probe to normalize the amount of RNA.

N18TG2 and NG108-15 total RNAs were also used in RT-PCR using MI and MII as primers, to confirm the presence of the PDE5 isoform in rodent neuronal cell lines. In both cases an amplified fragment of 792 bp was obtained, showing the same electrophoretic mobility as the bovine lung amplified fragment (Fig. 1A). Restriction analysis of the neuroblastoma amplification products gave the same results than bovine lung products, indicating a substantial homology of neuroblastoma and bovine lung PDE5 sequence.

Mouse N18TG2 RNA was also used in RT-PCR to amplify another PDE5 region using two alternative primers, MCs and MCa, highly conserved in the bovine and rat sequence (see Section 2). A cDNA product of the expected length (517 bp) was obtained (Fig. 1B) and cloned into pGEX-3X (MCPDE5N18). In both the bovine and rat lung PDE5 sequence, the 517 bp fragment encodes 171 amino acid residues in the N-terminal, 32 amino acids of the unique PDE5 sequence, and 139 amino acids of the cGMP-binding site [23,20]. Also in this case restriction analysis confirmed a substantial homology of mouse neuroblastoma RT-PCR product with bovine and rat lung sequences.

3.2. *Expression of PDE5 mRNA upon dbcAMP-induced differentiation of N18TG2 and NG108-15 cells*

To analyze the PDE5 mRNA level in neuroblastoma cells, PDE5 cDNAs from both bovine lung (MPDE5BL) and mouse neuroblastoma (MCPDE5N18) were used as probes in Northern blots of total RNA extracted from dbcAMP-treated and undifferentiated N18TG2 and NG108-15 cells. Using the bovine lung probe (MPDE5BL), a specific ~9 kb signal was revealed in bovine lung as well as in N18TG2 and NG108-15 (Fig. 2A). The mouse neuroblastoma probe (MCPDE5N18) recognized the same ~9 kb transcript (Fig. 3A). Both probes revealed an increase of PDE5 mRNA in NG108-15 cells as a result of dbcAMP treatment, while no evident change was observed in N18TG2 cells. Densitometric analysis was performed on the ~9 kb bands to quantify the level of PDE5 mRNA under the different culture conditions.

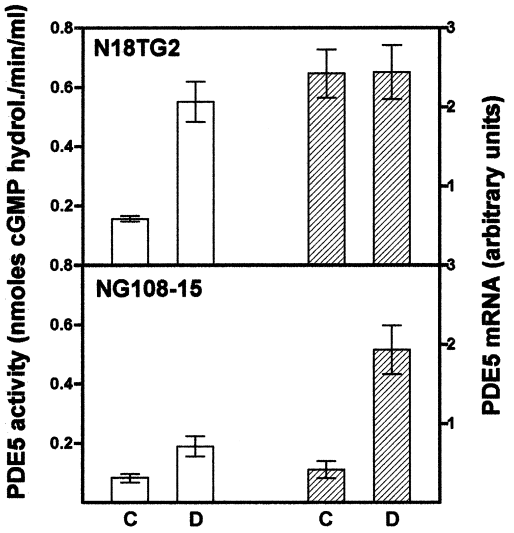

Fig. 4. dbcAMP induction of PDE5 mRNA compared with those of PDE5 activity in both N18TG2 and NG108-15 cell lines. Comparison between PDE activity (empty bars) and PDE5 mRNA expression (full bars) in N18TG2 cells (upper panel) and NG108-15 cells (lower panel). Each bar is the mean of six observations (±S.E.). C: control culture. D: 5 days dbcAMP treated culture.

Normalization to the house-keeping gene GAPDH mRNA (Fig. 2B, Fig. 3B) showed a 4.9-fold increase with respect to control cells in NG108-15 (Fig. 4). No significant increase of the PDE5 signal was observed in N18TG2 cells, although the induction of enzymatic activity in these cells was even higher than in NG108-15 (Fig. 4).

4. Discussion

RT-PCR analysis has shown a substantial homology of the neuroblastoma and bovine lung PDE5 sequence [23], as indicated by the same size (Fig. 1A) and restriction sites of the amplified bands from neuroblastoma and lung mRNA. In Northern blot, the probes obtained from bovine lung and neuroblastoma cells in fact recognize the same transcript.

Both mouse and bovine cDNA probes were then used to establish whether the differentiating agent dbcAMP elicited a higher transcription of the PDE5 gene, as suggested by the timing of enzyme activity induction [18].

Northern blot analysis demonstrates that a 5-fold higher level of PDE5 mRNA is found in dbcAMP treated NG108-15 cells, providing the first evidence of a cAMP-dependent induction of PDE5 expression. Until now only a cAMP-dependent short term mechanism of PDE5 activation due to PKA phosphorylation has been reported [35]. Recently it has been shown that in airway smooth muscle cells PKA-dependent activation of PDE5 can be prevented by the PDE6 γ inhibitory subunit (PDE6γ). Two small proteins, immunologically related to PDE6γ, were found to be associated to PDE5 and they were proposed as possible PDE5 regulatory subunits [36,41]. A similar mechanism may be operating in N18TG2 cells, where no increase in PDE5 mRNA level has been observed. However, the timing of enzyme induction in N18TG2 cells, which is not rapidly attained [18], is not consistent with a post-translational regulation of the enzyme. It may be therefore proposed that in these cells cAMP-induced activation of PDE5 requires the transcriptional regulation of an unknown regulatory element, possibly of the type described in airway smooth cells [36].

In conclusion, modulation of the PDE5 activity in the two cell lines appears to be dependent on the same signal, cAMP, although two distinct mechanisms seem to be operating: direct activation of PDE5 transcription in NG108-15, which is not observed in N18TG2. It is conceivable that the different behavior of the two cell lines is related to their different ability to establish synaptic contacts [39]. In rat cerebellum, transcriptional regulation of PDE5 during early postnatal development has been reported and increased PDE5 expression has been related to the development of cerebellar neuronal networks [20]. Thus our findings of a cAMP-induced transcriptional regulation of PDE5 in NG108-15 and its absence in N18TG2, may be consistent with a correlation between induction of PDE5 transcription and establishment of synaptic contacts. Furthermore, our findings together with the reported activation of PDE expression in rat cerebellum [20] and rat lung [42] suggest that activation of PDE5 expression may be a general phenomenon occurring during development and is related to cell differentiation and/or morphogenetic events in various tissues.

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