Volume 324, number 1, 76–80 FEBS 12553 June 1993

Characterization of 3′:5′ cyclic nucleotide phosphodiesterase activities of mouse neuroblastoma N18TG2 cells

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Received 4 March 1993; revised version received 12 April 1993

Characterization of 'low K_m' 3′:5′ cyclic nucleotide phosphodiesterase activities (PDE) expressed in mouse N18TG2 neuroblastoma cells is reported. At least 3 peaks of activity were isolated by DEAE chromatography, none of which was calcium-calmodulin stimulated and cGMP stimulated or inhibited. A first peak elutes at 200 mM sodium acetate; it specifically hydrolyzes cGMP with a K_m of 4.7 μM and shows sensitivity to zaprinast [M&B 22948] (1.8 μM). A second peak eluting at 410 mM sodium acetate hydrolyzes both cyclic nucleotides. A third peak, specific for cAMP hydrolysis, elutes at 580 mM sodium acetate, has a K_m of 3.2 μM and is sensitive to RO 20 1724 (7.6 μM) and rolipram (2 μM). Hydrodynamic analysis showed for the first peak a Stokes radius of 5.3 nm with a sedimentation coefficient of 8.1 S, a frictional ratio (f/f_0) of 1.41 and a native molecular mass of 182 kDa. The same analysis for peak 3 showed a Stokes radius of 4.1 nm with a sedimentation coefficient of 3.2 S, a frictional ratio of 1.63 and a native molecular mass of 56 kDa. The biochemical features reported for the enzyme eluting in the first peak, and its cGMP-binding activity stimulated by inhibitors of phosphodiesterase activity, demonstrate that it belongs to the PDE V subfamily; on the other hand the cAMP specific enzyme eluting in the third peak can be assigned to the 'RO 20 1724 inhibited' form. The significance of these findings is discussed in relation to the functional characteristics of the N18TG2 cell line.

High affinity cyclic nucleotide phosphodiesterase; PDE V subfamily; Neuroblastoma cell; Imidazolinone; Zaprinast

1. INTRODUCTION

Cyclic nucleotide phosphodiesterase (PDE) comprise a complex and growing group of distinct enzymes that differ in molecular size and charge, affinity for substrates and pharmacological inhibitors, response to modulators, subcellular localization and distribution among cells and tissues. PDEs are a main component of the second messenger system and thus are thought to be generally involved in the control of signal transduction. Little is known, however, on the specific role of different PDE isoforms and on the mechanisms that control their expression in different cell types and their functional correlation. Their ability to interact with a large number of effectors, which can differentially modulate the enzymatic properties of various isoenzymes, suggests that they may play an important role as mediators of a variety of intracellular signals and in particular of their integration [1].

This kind of function would be of particular relevance in the nervous system. The nervous system is characterized by high PDE activity; most of the known isoforms are present [2]. Immunolocalization studies have shown selective expression of the calcium-calmodulin activated isoenzyme in specific neurons and its subcellular localization in dendrites has suggested its relation to postsynaptic signal transduction [3,4]. Little is known about other PDE isoforms; it is conceivable that they play distinct roles in specific neuronal populations and/or in cellular functions independent of calcium flux.

For a better understanding of the functional role of the various PDE isoenzymes it would be useful to known in more detail their distribution in different regions of nervous system, their subcellular localization and their modifications in relation to different physiological and developmental states.

Cell heterogeneity of the nervous system seriously hampers this type of study and makes it necessary to use model systems, such as neuronal cell lines, which provide homogeneous cellular populations. Neuroblastoma clones have been widely used to investigate several aspects of neuronal function [5,6]. In fact, different clones express different specific neuronal properties, which can, moreover, be modulated by varying the culture conditions; this allows to establish possible correla-

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Abbreviations PDE, phosphodiesterase; DEAE, diethylaminoethyl; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FPLC, fast performance liquid chromatography; PMSF, phenylmethylsulfonylfluoride; IBMX, isobutylmethylxantine; zaprinast (M&B 22948), 1,4-dihydro-5-(2-propoxyphenyl)-7H-1,2,3-triazolo[4,5-d]-pyrimidin-7-one; RO 20-1724, 4-[(3-butyoxy-4-methoxyphenyl)methyl]-2-imidazolidinone; rolipram (ZK 62711), 4-[3-cyclopentyloxy-4-methoxyphenyl]-2 pyrrolidone; NBT, Nitrobleu tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate

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tions among the expression of neuronal components and specific functions. Thus the characterization of various PDE isoforms in neuroblastoma clones may provide interesting contributions for the understanding of their specific role in the regulation of neuronal functions.

The presence of PDE activity in neuroblastoma cells has been previously reported [5,7,8]. However, no data are available at present on the presence of various isoforms. We report here the characterization of cAMP-cGMP phosphodiesterase activities in the N18TG2 cell line. DEAE chromatography has allowed the identification of at least 2 distinct PDE activities. The first is a cGMP-specific isoenzyme, inhibited by zaprinast, which shows biochemical features very close to those described for the PDE V subfamily. The second specifically hydrolyzes cAMP and is inhibited by RO 20-1724 and rolipram.

2. MATERIAL AND METHODS

2.1 *Materials*

[^{3}H]cAMP (specific activity 28 Ci/mmol) was obtained from Amersham, [^{3}H]cGMP (specific activity 32.8 Ci/mmol) from New England Nuclear RO 20-1724 was a generous gift from Hoffman La Roche, rolipram (ZK 62711) from Schering and zaprinast (M&B 22948) from Rhône-Poulenc Rorer. Leupeptin, bestatin, pepstatin, calmodulin and anti-rabbit antibody alkaline phosphatase conjugate were obtained from Sigma Chemical Co. Bovine brain calcium-calmodulin PDE was purified as described [9]. All other reagents, purchased from various sources, were of analytical grade. Culture media and sera were obtained from Flow.

2.2. *Cells cultures*

Murine neuroblastoma cells, N18TG2, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (5,000 IU/ml), streptomycin (5 mg/ml) and supplemented with 10% fetal calf serum (FCS). 5×10^5 cells were plated on 90 mm culture dishes (Falcon) with 10 ml medium and grown in a humidified atmosphere of 10% CO_2 at 37°C. Confluent cultures (5×10^6 cells) were washed 2 times with phosphate-buffered saline (PBS) and the cells removed with 2 ml of PBS containing 0.1 M EDTA. Cells were then collected by centrifugation at $800 \times g$ for 10 min, frozen in liquid nitrogen and stored at −80°C.

2.3. *Enzyme extract*

Cells were suspended in homogenization buffer (20 mM HEPES pH 7.2, 0.2 mM EGTA, 5 mM β-mercaptoethanol, 10 μg/ml leupeptin, 5 μg/ml bestatin, 5 μg/ml pepstatin A, 1 mM PMSF, 0.1% Triton X-100) and homogenized at 4°C in a glass-teflon homogenizer with 15 strokes. The homogenate was centrifuged for 45 min at $100,000 \times g$, the pellet was resuspended in homogenization buffer and centrifuged for 45 min at $100,000 \times g$. The first and second supernatants were then pooled together. The same procedure was followed in cGMP-binding experiments, except for homogenization buffer (10 mM phosphate buffer pH 7.4, 1 mM EDTA, 12 mM β-mercaptoethanol).

2.4. *DEAE chromatography*

The supernatant was diluted 1:2 with 50 mM sodium acetate pH 6.5, 0.2 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 5 mM NaF, 0.05% Triton X-100 (buffer A) and loaded onto a 5 ml DE52 cellulose (Whatman) column equilibrated with the same buffer. After loading the column was washed with 5 bed volumes of buffer A and PDE activity was eluted with 50 ml of a linear 50–1,000 mM sodium acetate gradient in buffer A, collecting 0.9 ml fractions. Fractions corresponding to peaks of enzyme activity were pooled and concentrated with centricon-10 membranes (Amicon) for further analysis.

2.5 *Analytic gel filtration by FPLC*

Aliquots of the pooled fractions were loaded onto a FPLC Superose 12 HR 10/30 (Pharmacia) column and eluted at a flow rate of 250 μl/min with 20 mM HEPES pH 7, 1 mM EGTA, 5 mM β-mercaptoethanol, 5 mM $MgCl_2$, 100 mM NaCl, 2 mM PMSF, 0.05% Triton X-100.

2.6. *Sucrose density gradient*

Aliquots of pooled and concentrated fractions were layered onto a 12 ml linear 5–20% sucrose gradient in 50 mM sodium acetate pH 6.5, 0.2 mM EGTA, 5 mM $MgCl_2$, 0.2 mM PMSF and centrifuged in a Beckman SW40 rotor for 20 h at $150,000 \times g$ at 4°C. 0.3 ml fractions were collected and aliquots assayed for PDE and sedimentation standard activities.

2.7. *Enzymatic assay*

PDE activity was determined with the two step method as described [10], 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) with a substrate concentration of 1 μM, unless otherwise indicated. Calmodulin stimulation experiments were done using 1 mM $CaCl_2$ and 3 μg/ml of calmodulin.

2.8 *Cyclic GMP binding assay*

cGMP binding was measured on cell extracts, using 0.5 μM [^{3}H]cGMP in a total volume of 0.1 ml of 'cGMP binding buffer' (10 mM phosphate buffer pH 7.4, 1 mM EDTA, 12 mM β-mercaptoethanol, 10 μM cAMP, 0.5 mg/ml BSA). The reaction was started by the addition of cell extract; after 120 min in an ice-bath the assay mixtures were filtered onto HAWP filters (Millipore) which were washed three times with 10 mM phosphate buffer pH 7.4, 1 mM EDTA. After drying the filters were counted in 10 ml of membrane filters scintillation cocktail (complete LSC; Packard Instruments Co.).

2.9 *Immunoblot*

SDS-gel electrophoresis was performed on 8% slab gels [11] and proteins transferred to nitrocellulose membranes as described [12]. The blots were then incubated overnight at 4°C with rabbit affinity purified polyclonal antibody against bovine brain calcium-calmodulin dependent PDE diluted 1:1,000. The blot was then incubated 1 h with anti-rabbit antibody conjugated to alkaline phosphatase. The immunocomplexes were visualized by NBT-BCIP system. Bovine brain calcium-calmodulin PDE was used as a positive control.

2.10 *Other procedures*

Protein concentration was determined as described [13] using BSA as standard. Stokes radius [14], native molecular mass and frictional ratio [15] were calculated as previously described.

3. RESULTS

3.1. *Enzyme extraction*

Extraction of cyclic nucleotide phosphodiesterase activity from N18TG2 neuroblastoma cells is partly dependent on the presence of Triton X-100; in fact in the absence of Triton X-100 only 69% of the cAMP and 80% of the cGMP hydrolytic activity was recovered, while using 0.1% Triton X-100, recovery was 96% and 100%, respectively, for cAMP and cGMP activity. The specific activity of the enzyme extracted in the presence of Triton X-100 is, respectively, 0.18 nmol of cAMP hydrolyzed/min/mg protein and 0.15 nmol of cGMP hydrolyzed/min/mg protein.

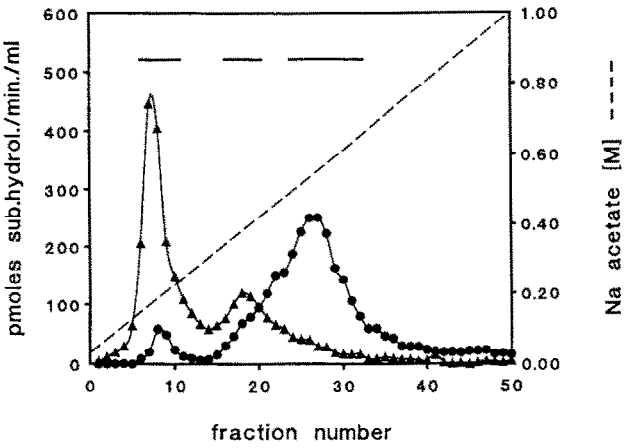

Fig. 1. DEAE-cellulose chromatography of N18TG2 neuroblastoma extract. See section 2 for details of the procedure. Phosphodiesterase activity was assayed in the presence of EGTA with 1 μM cGMP (▲) and with 1 μM cAMP (●) as substrates. Horizontal segments above profile indicates pooled fractions.

3.2. *Isoform separation and characterization*

The DEAE-chromatography elution profile (Fig. 1) shows the presence in these neuroblastoma cells of at least 3 peaks of PDE activity, eluting, respectively, at 200 mM (peak 1), 410 mM (peak 2) and 580 mM (peak 3) sodium acetate. Only a minor fraction (about 7%) of total cAMP activity was found in peak 1, while the large majority was associated with peak 3; on the contrary 70% of cGMP hydrolytic activity was eluted in peak 1 and 30% in peak 2. Recovery of hydrolytic activity was 80–90% in all chromatographic runs. To further characterize the kinetic and biochemical properties of the separated isoforms, fractions corresponding to peaks 1, 2 and 3, were pooled as indicated by the horizontal bars in Fig. 1. The activity present in all peaks was found independent of calcium-calmodulin; it was also neither stimulated nor inhibited by cGMP in a concentration range of 0.1–10 μM. The absence of calcium-calmodulin isoform was also observed in the cell extract; it was further confirmed by Western blot analysis, using an affinity purified polyclonal antibody against bovine brain isoform.

Table I

Biophysical properties of two phosphodiesterase isoform present in N18TG2 cells and comparison with PDE V subfamily isoforms*

	Rat lung [13]	Rat platelets [16]	N18TG2	
			1	3
Stokes radius (nm)	5.5	5.6	5.3	4.1
Sedimentation coefficient (S)	7.8	6.4	8.1	3.2
Native molecular mass (kDa)	177	176	182	56
Frictional ratio (f/f_0)	1.54	1.48	1.41	1.63
K_m	4.0	ND	4.7	3.2

*All values were obtained using cGMP as substrates except for the neuroblastoma peak 3 values where cAMP was used.

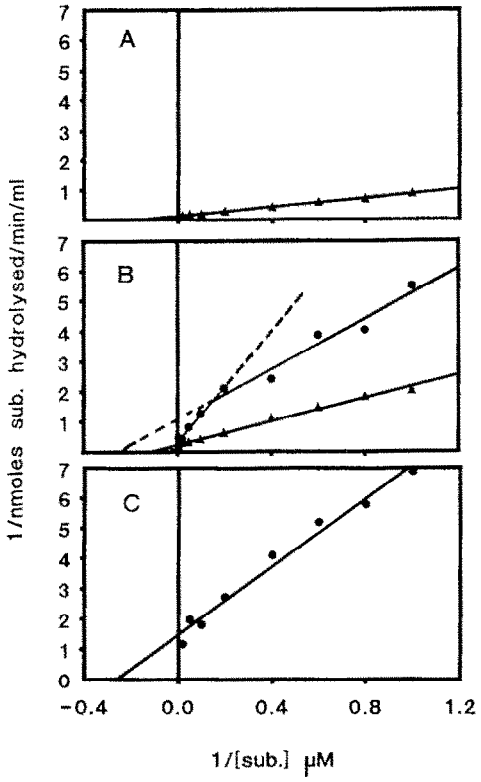

Fig. 2. Lineweaver–Burk plots of substrate hydrolysis by pooled fractions from PDE activity peaks separated by DEAE-cellulose chromatography: (A) peak 1, (B) peak 2, (C) peak 3. Phosphodiesterase activities were assayed in the presence of EGTA with cGMP (▲) and cAMP (●) as substrates.

Fig. 2 shows the kinetic analysis in a concentration range 1–100 μM of cyclic nucleotides. The activities present in peak 1 and 3 show in Lineweaver–Burk plots a linear relation with substrate concentration; peak 1 PDE has a K_m of 4.7 ± 1.25 μM for cGMP and peak 3 a K_m of 3.2 ± 1.1 μM for cAMP; on the other hand peak 2 displays a linear relation when cGMP is used as substrate, with a K_m of 8.8 ± 0.9 μM and a biphasic behaviour for cAMP with two K_m values of 4 ± 0.72 and 30 ± 2.8 μM, respectively.

Sucrose density gradient analysis shows a sedimentation constant of 8.1 S for peak 1 PDE and 3.2 S for peak 3. FPLC gel-filtration analysis indicates a Stokes radius of 5.3 nm for peak 1 and 4.1 nm for peak 3. As reported in Table I the values of molecular mass and frictional ratio calculated from the above data are respectively 182 kDa and 1.41 for peak 1 and 56 kDa and 1.63 for peak 3.

3.3. *Inhibition and binding analysis*

The action of well known inhibitors of PDEs was also

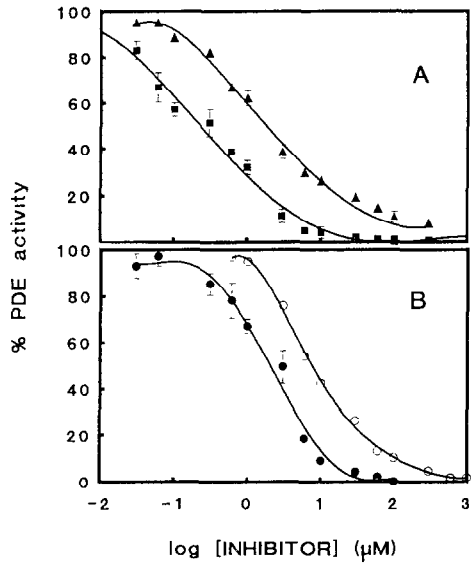

Fig. 3. (A) Inhibition by zaprinast (M&B 22948) of PDE eluted in peak 1 (▲) and peak 2 (■). PDE activity was assayed with 1 μM cGMP as substrate. (B) Inhibition by RO 20-1724 (○) and rolipram [ZK 62711] (●) of PDE eluted in peak 3. PDE activity was assayed with 1 μM cAMP as substrate.

assayed on the pooled and concentrated fractions corresponding to the isolated peaks. Zaprinast exhibits a high inhibitory effect on peak 1 and peak 2 activity, with a K_i of 1.2 μM and 0.3 μM, respectively (Fig. 3A). On the other hand only peak 3 is significantly inhibited by RO 20 1724, with a K_i of 7.6 μM and by rolipram with a K_i of 2 μM (Fig. 3B).

To further characterize PDE activity of N18TG2 cells, cGMP binding was also measured on total cell extracts, as described under experimental procedures. A cGMP binding of 1.07 pmoles of cGMP bound per mg of protein was measured. This activity was also found to be increased by a factor of 2 and 2.4, respectively, in the presence of 1 mM IBMX and 0.6 mM zaprinast (Fig. 4).

4. DISCUSSION

Neuroblastoma N18TG2 cells contain 'low K_m' cAMP and cGMP PDE activities. Requirement of Triton X-100 for solubilization is different for the two activities, indicating a higher degree of hydrophobicity for cAMP-specific PDE, which not only is extracted at a lower extent in the absence of Triton X-100 as compared to cGMP PDE, but also needs 0.05% Triton X-100 in all performed chromatographic separations.

On the basis of DEAE elution profile, hydrodynamic and kinetic properties and inhibition studies we have demonstrated the presence of at least 3 distinct peaks, each one using as substrate either cAMP or cGMP; all three enzymes are neither stimulated by cGMP or calcium-calmodulin nor inhibited by cGMP.

cGMP hydrolytic activity eluting at the lowest ionic strength (Fig. 1) on the basis of its K_m, sedimentation constant, native molecular mass and Stokes radius values can be designated as a member of the 'cGMP binding-cGMP specific PDE' family, characterized in rat lung [16,17], bovine lung [18] and rat platelets [19,20] (Table I). Further data in favour of this assignment are zaprinast inhibition [21] and cGMP binding activity, shown respectively in Figs. 3 and 4. Moreover this isoenzyme displays the property that IBMX or zaprinast inhibition of PDE catalytic site stimulates cGMP binding activity [22,23]. These features together with the absence of effect on enzyme activity by cGMP and calmodulin indicate that peak 1 cGMP PDE belongs to PDE V family. This PDE isoform is not the only one present in peak 1. In fact the coeluting cAMP hydrolytic activity can be separated by gel filtration and sucrose gradient from cGMP peak 1 activity.

cGMP PDE isoforms eluted in peak 1 and 2 differ for their K_m values and zaprinast inhibition; however their hydrodynamic features (data not shown), as well as their substrate specificity are very similar. The two enzyme molecules thus appear as closely related proteins. Their different behaviour in DEAE chromatography may be dependent on posttranslational modifications of a single protein or to a rather stable binding with some small molecules, as shown for the cGMP-PDE from bovine lung [18]. PDE activity eluting in peak 3 specifically hydrolyzes cAMP; this characteristic together with its hydrodynamic and inhibition properties allows to classify it as 'low K_m' RO 20 1724 inhibited form' [24,25]. This PDE family is widely distributed in several tissues and has been studied in details in mammalian liver [26], kidney [27], testis [28] and brain [29]. In all these tissues a certain degree of hydrophobicity and molecular asymmetry, resulting in a particularly high frictional ratio, is evident; similar characteristics are found in N18TG2 cells (Table I). The shape of peak 3 suggests that this activity is not due to a single enzyme but that several related isoforms, eluting in a very close

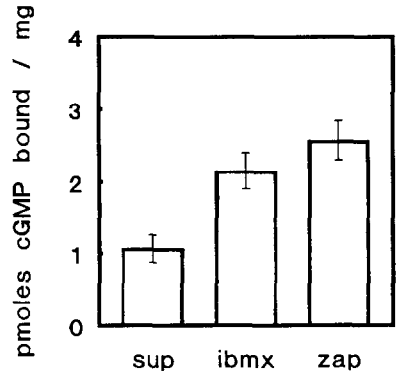

Fig. 4. Effects of PDE inhibitors on cGMP binding; sup, neuroblastoma supernatant binding , mix, binding in the presence of 1 mM IBMX, zap, binding in the presence of 0.6 mM of zaprinast.

range of ionic strength, are present. Indirect evidence for the heterogeneity of this PDE family is provided by gene cloning analysis from mammalian tissues, which has isolated four cDNAs corresponding to distinct but homologous genes; transcripts of three of these genes are found in high amount in brain [30,31].

In conclusion from the studies reported here on the characterization of PDEs in N18TG2 neuroblastoma clone two findings appear particularly relevant: the absence of calcium-calmodulin dependent PDE isoforms, which are predominant in brain, and the presence of cGMP PDE V. These differences of N18TG2 PDE pattern, as compared with brain, are not surprising. In fact it is known that calmodulin-dependent PDE is not ubiquitously distributed in all central neurons [4]; furthermore it has not been found in sympathetic neurons of the superior cervical ganglia [32]. It should be noted that N18TG2 is the first cell line so far demonstrated to express high level of PDE V; it therefore can be an interesting model to investigate PDE V role in cGMP metabolism and of neuronal subpopulations not expressing calmodulin dependent PDE isoforms; it thus could be particularly suitable for a better characterization of PDE V properties and functions in relation to neuron specificity and possibly differentiation.

Acknowledgements. We express our thanks to Prof S. Biagioni for helpful discussion and comments on the manuscript. We are also grateful to La Roche for generous gifts of RO 20 1724, Schering for rolipram, Rhône Poulenc Rorer for zaprinast and Dr. Randall Kincaid for antibody against the calmodulin-stimulated isoform. This work was supported by MURST 40% and 60% grants.

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