Cyclic nucleotide phosphodiesterases in somatic and germ cells of mouse seminiferous tubules*

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Summary. The distribution of phosphodiesterase forms in somatic and germ cells, and their variations during testicular development and germ cell differentiation have been investigated. Seminiferous tubules from immature mice and Sertoli cells in culture possessed two enzyme activities which were comparable to forms described for different tissues and species: (a) a calcium–calmodulin-dependent enzyme with high affinity for guanosine 3',5'-(cyclic)-monophosphate (cGMP), and (b) a calcium–calmodulin-independent enzyme with high affinity for adenosine 3',5'-(cyclic)-monophosphate (cAMP) the activity of which increased in cultured Sertoli cells after treatment with FSH or dibutyryl cAMP. Seminiferous tubules from adult animals and germ cells at the meiotic and post-meiotic stage of differentiation possessed two enzyme forms that could be distinguished from those present in somatic cells of the seminiferous tubules: (a) a calcium–calmodulin-dependent form with high affinity for both cAMP and cGMP, similar to forms described in other tissues from different species, and (b) a calcium–calmodulin-independent phosphodiesterase with high affinity for cAMP and present only in post-meiotic cells, previously identified also in germ cells of the rat.

Introduction

It is well documented that cyclic nucleotides play multiple roles in the regulation of testicular function. In somatic cells they mediate Sertoli and Leydig cell response to gonadotrophins (as reviews see: Means *et al.*, 1980; Purvis & Hansson, 1980). At the level of the germ cells, they are thought to be involved in the control of sperm maturation in the epididymis (see Sanborn, Heindel & Robison, 1980, for review). Furthermore, the observation that high levels of cAMP cause a reduction in spermatogonial divisions (Hollinger & Hwang, 1974) and inhibition of spermiation (Gravis, 1978) suggests a participation of cyclic nucleotides in the control of male germ cell differentiation in the seminiferous tubules either acting directly within the germ cells or via the mediatory role of Sertoli cells. The hypothesis that cyclic nucleotides participate in the control of germ cell differentiation is further strengthened when it is considered that high cAMP concentration is required to prevent resumption of meiosis in amphibian and mammalian oocytes (Morrill, Zeigler & Kostellow, 1981; Schultz, Montgomery & Belanoff, 1983) and to prevent the onset of meiosis in yeast (Matsumoto, 1983).

Cyclic AMP level in the seminiferous tubules is regulated in a complex fashion as indicated by studies in different cell types. It varies in germ cells at different stages of differentiation (Spruill & Steiner, 1976; Adamo, Conti, Geremia, Boitani & Monesi, 1980a) and in Sertoli cells, according to the age of the animal (Means *et al.*, 1980) or to the refractory state after FSH treatment (Verhoeven,

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Cailleau & De Moor, 1980; Conti, Toscano, Petrelli, Geremia & Stefanini, 1983b). It has been suggested that intracellular levels of cAMP are regulated in germ cells by changes of phosphodiesterase activity (Geremia, Rossi, Pezzotti & Conti, 1982) on the basis of the observation that these cells possess an adenylate cyclase activity which is Mn^{2+} -dependent, and NaF- and hormone-insensitive (Braun, 1974; Adamo, Conti, Geremia & Monesi, 1980b; Gordeladze & Hansson, 1981), and whose activity does not correlate with the level of cAMP (Adamo *et al.*, 1980a). The importance of catabolism in the regulation of cAMP levels in Sertoli cells has been emphasized: phosphodiesterase variations would be involved in the age-dependent modifications of the response to FSH and in the development of a refractory state after hormone stimulation (Means, Dedman, Tindall & Welsh, 1978; Spruill, Steiner, Tres & Kierszenbaum, 1981; Conti, Geremia, Adamo & Stefanini, 1981; Verhoeven *et al.*, 1981; Conti *et al.*, 1982, 1983a, b).

The present study on phosphodiesterase forms in mouse testis has been performed with the aim of defining their distribution in somatic and germ cells, their variation during testicular development and germ cell differentiation, and the mechanisms of their regulation. A preliminary report of this work has been published in abstract form (Geremia, Mocini, Pezzotti, Conti & Rossi, 1983a).

Materials and Methods

Biological materials. Male Swiss CD1 mice were used in all experiments. Seminiferous tubules were freed of interstitial tissue by collagenase treatment at room temperature in Dulbecco's phosphate-buffered saline (PBS) followed by several washes by sedimentation in PBS (Geremia, D'Agostino & Monesi, 1978). Total germ cell suspensions were obtained by trypsin treatment of seminiferous tubules from 60-day-old animals (Geremia *et al.*, 1978). Germ cells at defined stages of differentiation were obtained by sedimentation velocity fractionation at unit gravity in albumin gradients of the cell suspensions (Lam, Furrer & Bruce, 1970). Germ cell fractions (80–90% homogeneous) containing middle-late pachytene spermatocytes, round spermatids at steps 1 to 8 of spermiogenesis and intermediate spermatids at steps 9 to 13 of spermiogenesis were used. Cell fractions were washed twice in PBS before use. Sertoli cell cultures from 20-day-old mice were performed as previously described for the rat (Dorrington, Roller & Fritz, 1975) and were maintained in Minimum Essential Medium with Earle's salts (Gibco, Paisley, Scotland) at 32°C in a controlled atmosphere of air and 5% CO₂. After 3 days of culture Sertoli cell monolayers were briefly exposed to 20 mM-Tris-(hydroxymethyl)-methylamine (Tris-HCl) pH 7.4 hypotonic solution to remove remaining germ cells (Galdieri, Ziparo, Palombi, Russo & Stefanini, 1981). At 24 h after germ cell removal, Sertoli cell monolayers were treated for 24 h with dibutyryl cyclic AMP (dbcAMP) (Sigma, St Louis, MO, U.S.A.) or FSH (NIADDK-ovine-FSH-S15). Spermatozoa were prepared from the caput and cauda epididymidis as described by Majumder (1978). Epididymides were cut with a razor blade and the fragments were gently stirred at room temperature for 30 min in PBS. The resulting suspension of spermatozoa was separated from epididymal fragments by sedimentation at unit gravity and then washed twice by centrifugation.

Cytosol preparation. Seminiferous tubules, germ cells, Sertoli cell monolayers or spermatozoa were homogenized at 4°C with 15 strokes in a Dounce homogenizer in 4–5 volumes of 20 mM-Tris-HCl, pH 8, 10 mM-2-mercaptoethanol, 2 mM-phenylmethylsulphonylfluoride and 0.2 mM-ethyleneglycolbis-(aminoethylether)-tetra-acetic acid (EGTA), unless otherwise stated in 'Results'. The homogenate was then centrifuged for 15 min at 20 000 *g* and 1 h at 105 000 *g*. The supernatant thus obtained is referred to as cytosol. Protein content was determined with a modification of the method of Lowry (Bensadoun & Weinstein, 1976).

Ion-exchange chromatography. DEAE-cellulose chromatography (DE52, Whatman) was performed at 4°C essentially as described elsewhere (Geremia *et al.*, 1982). Columns (0.6 × 10 cm)

were equilibrated with 70 mM-sodium acetate, pH 6.5, 5 mM-2-mercaptoethanol and 0.2 mM-EGTA. Cytosol containing 3–5 mg protein was applied to columns after a 1:4 dilution with equilibration buffer. After washing with 4 column volumes of the same buffer, elution was performed with a linear sodium acetate gradient (70–600 mM) containing 10 mM-2-mercaptoethanol and 0.2 mM-EGTA at a constant flow rate of 0.25 ml/min. Fractions of 0.5 ml were collected in tubes containing 50 μ l 0.1% bovine serum albumin. Recovery of activity ranged between 80 and 90%. Chromatographic separation of enzyme activity was performed in the presence of chelating agents to minimize the formation of calcium–calmodulin–enzyme complexes which would elute at a different ionic strength with respect to the free enzyme.

Phosphodiesterase assay. Enzyme activity was measured as conversion of cAMP and cGMP to their respective 5'-nucleotides, according to the method of Thompson & Appleman (1971). The incubation mixture, in a final volume of 200 μ l, contained 20 mM-Tris-HCl, pH 8, 5 mM-MgCl₂, 5 mM-2-mercaptoethanol, 0.1 mM-EGTA, 1 mM-CaCl₂, and, when stated in 'Results', 3 μ g purified calmodulin (Jamieson & Vanaman, 1979). Cyclic AMP and cGMP were used at a final concentration of 1 μ M (containing 100 000 c.p.m. [³H]nucleotide/tube, routinely purified by gel filtration). The reaction was performed at 34°C and stopped by adding 200 μ l 40 mM-Tris-HCl, pH 7.5 containing 10 mM-ethylenediaminetetra-acetic acid (EDTA) and boiling for 1 min. The second incubation with *Crotalus atrox* venom (Sigma) and separation of nucleosides by batch adsorption were as previously described (Thompson & Appleman, 1971). Enzyme concentration and time of reaction were adjusted so that no more than 20% of the substrates was hydrolysed. The assay condition to evaluate basal enzyme activity was chosen after preliminary experiments which showed that the addition of Ca²⁺ did not modify the enzyme activity profile obtained in the presence of EGTA, provided that chromatography had been performed in the presence of chelating agents. The calmodulin–enzyme complexes that are eluted from the column when Ca²⁺ is present during chromatography would be detected, in fact, as peaks of activity fully stimulated by the addition of Ca²⁺ alone to the assay mixture.

Results

Phosphodiesterase forms in testes from immature and adult mice

With the homogenization conditions employed, 90% of phosphodiesterase activity present in total homogenate was recovered in the cytosol, while 8% was present in the 20 000 g pellet and only a trace amount was found in the 100 000 g pellet.

Two main peaks of activity were resolved by ion-exchange chromatography of cytosol from 12-day-old mouse seminiferous tubules (Text-fig. 1a, b). The first peak eluting at 130 mM-sodium acetate hydrolysed cGMP better than cAMP, and the activity was stimulated by Ca²⁺ and calmodulin. A second peak of activity eluting at 430 mM-salt hydrolysed cAMP only, and the activity was not affected by calmodulin. A peak of activity hydrolysing mainly cGMP eluted at 250 mM-salt, and was almost insensitive to calmodulin stimulation (~30%). When chromatography was performed in the presence of 1 mM-CaCl₂, the activity eluting at 130 mM-salt was reduced while that eluting at 250 mM increased proportionally (data not shown). This indicated that the two activities represent the same enzyme form which shows different chromatographic behaviour when free or when bound to calmodulin, as described for the rat testis (Geremia *et al.*, 1982).

Two main peaks of activity were present in cytosol from 60-day-old mouse seminiferous tubules (Text-fig. 1c, d) which are different from those found in the immature testis. A first peak of activity eluting at about 110 mM-sodium acetate hydrolysed both cAMP and cGMP at the same rate, and was stimulated by Ca²⁺ and calmodulin. A second peak of activity eluting at 350 mM-sodium acetate hydrolysed cAMP only, and was insensitive to Ca²⁺ and calmodulin stimulation. Only trace amounts of cAMP hydrolysing activity could be detected at 430 mM salt concentration.

The variation of stimulation was within 10% in different experiments.

Text-fig. 1. Chromatographic patterns (representative of 3 different experiments) of phosphodiesterase activity from cytosol of seminiferous tubules from testes of 12-day-old and 60-day-old mice. Cyclic AMP (a, c) and cGMP (b, d) hydrolytic activity was measured in aliquants of the collected fractions in the presence of 1 μ M substrate and 1 mM- CaCl_2 (○) or 1 mM- CaCl_2 and calmodulin (●). See 'Materials and Methods' for details.

Kinetic properties

Lineweaver-Burk plots of substrate concentration studies of the calcium-calmodulin-dependent activity from immature mouse testis exhibited linear kinetics for both cAMP (Text-fig. 2a) and cGMP (Text-fig. 2b). The enzyme appeared to be a low-affinity form for cAMP (apparent K_m 20 μ M) and a high affinity form for cGMP (apparent K_m 2 μ M). Calmodulin enhanced V_{max} 4-fold without affecting K_m values (data not shown). To establish whether this enzyme form possesses distinct catalytic sites for the two substrates, the effect of different concentrations of the non-radioactive form of a nucleotide on three different concentrations of the labelled form of the other nucleotide was studied. Text-figure 3(a, b) represents Dixon plots ($1/v$ versus $[I]$) of the experimental data showing a competitive inhibition of cAMP versus cGMP ($K_i \sim 20 \mu$ M) and *vice versa* ($K_i \sim 2 \mu$ M), thus indicating that the two substrates are hydrolysed by the same catalytic site. Kinetic data on the calcium-calmodulin-dependent activity (110 mM-salt) from adult mouse testis have been reported elsewhere (Geremia, Rossi, Mocini, Pezzotti & Conti, 1984). The enzyme from the adult mouse testis possessed substrate specificity characterized by high affinity for cGMP ($K_m \sim 3.5 \mu$ M), and cAMP ($K_m \sim 2 \mu$ M) and low affinity for cAMP ($K_m \sim 20 \mu$ M), and possessed distinct catalytic sites that interacted allosterically. Therefore Ca^{2+} -dependent phosphodiesterases from adult and immature mouse testes are clearly distinct forms with regard to kinetic parameters.

Both calcium-calmodulin-independent forms showed specificity for cAMP which they hydrolysed with similar kinetics. The enzyme form from adult testis, eluting at 350 mM-salt concentration, showed a K_m of 5 μ M (Text-fig. 2c) and that from immature testis, eluting at 430 mM-salt, a K_m of 2 μ M (Text-fig. 2d). It was therefore possible to distinguish between these two forms on their different chromatographic behaviour.

Text-fig. 2. Lineweaver-Burk plots of substrate concentration studies of the calcium-calmodulin-dependent phosphodiesterase from 12-day-old mice (130 mM-salt) and the calcium-calmodulin-independent forms (430 mM- and 350 mM-salt) from 12-day- and 60-day-old mice, respectively. Pooled chromatographic fractions were used. The kinetic analysis of the calcium-calmodulin-dependent form from immature mice was performed with cAMP (a) and cGMP (b) as substrates, in the presence of 1 mM- CaCl_2 and calmodulin. The analysis of the calcium-calmodulin-independent forms from adult (c) and immature (d) mice was performed with cAMP as substrate. Each point was the mean of triplicate determinations from at least 2 experiments. The s.d. was within 10% of the mean value.

Text-fig. 3. Effect of different concentrations of the non-radioactive form of a nucleotide (abscissa) on the hydrolysis of the labelled form of the other (indicated in the panels) by the calcium-calmodulin-dependent phosphodiesterase (130 mM-salt) of testes of immature mice. Substrate concentrations were, 0.3 μM (○), 0.6 μM (●) and 1.2 μM (■). The reciprocal of enzyme activity measured in the presence of 1 mM- CaCl_2 and purified calmodulin is shown on the ordinate, by Dixon plot ($1/v$ versus $[I]$). Each point is the mean of triplicate determinations from 2 experiments. The s.d. was within 10% of the mean value.

Distribution of phosphodiesterase forms in germ cells and somatic cells of the seminiferous tubule

To verify whether the different enzyme composition in the testes of adult and immature mice is the result of a selective distribution of phosphodiesterase forms in different cell types of seminiferous tubules, Sertoli cell cultures and germ cells at defined stages of development were studied.

The chromatographic pattern of phosphodiesterase activity from cytosol of cultured Sertoli cells (Text-fig. 4a) was similar to that observed in seminiferous tubules from the immature gonad: furthermore, the activity eluting at 130 mM-salt hydrolysed cGMP only and was stimulated by calmodulin (data not shown) as in immature seminiferous tubules. The kinetics of enzyme activities from Sertoli cells were the same as those from immature gonads (data not shown). Experiments were also performed to verify whether the calcium-calmodulin-independent form of phosphodiesterase in mouse Sertoli cells is under FSH control as in the rat (Conti *et al.*, 1982). Cyclic AMP hydrolysis in crude homogenates increased about 2-fold after 24-h incubation of Sertoli cells with 500 ng FSH/ml while the calcium-calmodulin-dependent cGMP hydrolytic activity was unaffected (data not shown). The increase of enzyme activity seemed to be mediated by cAMP, because an augmentation of the specific activity of the calcium-calmodulin-independent form (430 mM-salt) was also obtained after incubation of Sertoli cells for 24 h with 1 mM-dbcAMP (Text-fig. 4b).

Text-fig. 4. Chromatographic patterns (representative of 2 different experiments) of phosphodiesterase activity from cytosol of control and dbcAMP stimulated Sertoli cell cultures. Cyclic AMP (●) and cGMP (○) hydrolytic activity was measured in (a) control cells and (b) after incubation for 24 h in the presence of 1 mM-dbcAMP using 1 μ M-substrate, in the presence of 1 mM- CaCl_2 and calmodulin.

Chromatographic analysis of phosphodiesterase activity from cytosol of germ cell-enriched populations at different stages of development (Text-fig. 5a-f) exhibited patterns essentially similar to those observed for adult seminiferous tubules. The kinetics of enzyme activities from enriched germ cells were the same as those from adult testis (data not shown). Nonetheless, the profile changed at different stages of spermatogenesis: the calcium-calmodulin-dependent cAMP-cGMP specific form (110 mM-salt) was present with the same specific activity in all differentiative stages considered; the calcium-calmodulin-independent cAMP specific form (350 mM-salt) was absent in meiotic stages (Text-fig. 5a), appeared and reached maximal specific activity in round spermatids (Text-fig. 5c) and declined in late spermiogenesis (Text-fig. 5e).

Text-fig. 5. Chromatographic analysis of phosphodiesterase activity from cytosol of germ cell-enriched populations at different stages of development (patterns representative of 3 different experiments). The enzyme activity in middle-late pachytene spermatocytes (a, b), round spermatids (c, d) and elongating spermatids (e, f) was measured using $1\ \mu\text{M}$ -cAMP (a, c, e) and $1\ \mu\text{M}$ -cGMP (b, d, f) as substrate, in the presence of CaCl_2 alone (○) or CaCl_2 and calmodulin (●).

In addition, to establish whether the phosphodiesterase activities identified in germ cells can be found in other cell types of the mouse, chromatographic analyses of cytosol from spleen, brain, pancreas, kidney and heart were performed. None of the tissues showed a calcium-calmodulin-dependent cAMP-cGMP high-affinity phosphodiesterase or a calcium-calmodulin-independent cAMP-specific enzyme that eluted at 350 mM-salt concentration (data not shown).

Phosphodiesterase forms in epididymal spermatozoa

Chromatographic analysis of cytosol showed the same enzyme activities present in differentiating germ cells, but the activity ratio between the two forms was changed, being approximately 1:1 (Text-fig. 6). The activity eluting at 110 mM-salt was stimulated 2.5-fold by calmodulin (data not shown). Phosphodiesterase activity in epididymal spermatozoa appeared to be mainly present in the particulate fraction (70–80% of total activity), unlike the observations for seminiferous tubules, Sertoli cells and differentiating germ cells. Most of the activity could be extracted by adding Triton X-100 at a final concentration of 1% to the homogenization buffer (data not shown).

Text-fig. 6. Chromatographic analysis of phosphodiesterase activity from cytosol of epididymal spermatozoa (patterns representative of 2 different experiments). The activity was measured using $1\ \mu\text{M}$ -cAMP (●) or $1\ \mu\text{M}$ -cGMP (○) as substrates, in the presence of $1\ \text{mM}$ - CaCl_2 and calmodulin.

Discussion

We have identified four phosphodiesterase forms in the mouse testis which can be distinguished on the basis of chromatographic behaviour and kinetic properties, and which display a distinct distribution in somatic and germ cells.

Somatic cells of the seminiferous tubules possess a calcium-calmodulin-dependent high-affinity cGMP phosphodiesterase eluting at $130\ \text{mM}$ -sodium acetate and a calcium-calmodulin-insensitive high-affinity cAMP enzyme eluting at $430\ \text{mM}$ -sodium acetate. They are the main forms found in seminiferous tubules from immature testes and in Sertoli cell-enriched cultures, while their activity is greatly reduced in the adult testis and absent in germ cell enriched preparations. The calcium-calmodulin-dependent enzyme activity is similar to that purified from bovine brain (Ho, Wirch, Stevens & Wang, 1977; La Porte, Toscano & Storm, 1979) and heart (Klee, Crouch & Krinks, 1979; Sharma, Wang, Wirch & Wang, 1980) and to a form described and characterized from rat testis (Geremia *et al.*, 1982). This activity can be distinguished from the calcium-calmodulin-dependent form present in germ cells (Geremia *et al.*, 1984). Substrate competition studies indicate, by the reciprocal competitive inhibition of cAMP and cGMP, that the somatic cell form possesses a single catalytic site. However, the germ cell form possesses multiple hydrolytic sites in which cGMP and cAMP acted as non-competitive reciprocal inhibitors (Geremia *et al.*, 1984). The calcium-calmodulin-independent phosphodiesterase of somatic cells ($430\ \text{mM}$ -salt) shows marked similarities with the hormone-regulated enzymes described for different tissues (for review, see Wells & Hardman, 1977) and resembles an analogous form identified in Sertoli cells of the rat. The enzyme activity described herein displays the same chromatographic behaviour and kinetic properties as the rat enzyme and it can be also stimulated by exposure of mouse Sertoli cells in culture to FSH or dbcAMP; in the rat, this enzyme activity has been shown to be under FSH control both *in vitro* (Conti *et al.*, 1981; 1982) and *in vivo* (Conti *et al.*, 1983a).

Mouse germ cells possess a calcium-calmodulin-dependent phosphodiesterase with high affinity for cGMP ($110\ \text{mM}$ -salt) and high and low affinity for cAMP (Geremia *et al.*, 1984), and a calcium-calmodulin-independent phosphodiesterase with high affinity for cAMP ($350\ \text{mM}$ -salt). These enzymes are found in seminiferous tubules from adult mouse testis and in enriched germ cell populations, while they are almost absent in seminiferous tubules from immature testis and in Sertoli cells in culture. Furthermore, these forms are absent in extracts of other mouse organs. The calcium-calmodulin-dependent cAMP-cGMP enzyme activity has been characterized elsewhere (Geremia *et al.*, 1984) and it can probably be compared to similar forms described for different tissues from other species (see Beavo *et al.*, 1982, for review). In the homogenization conditions

used in this study most of the activity appeared to be in the cytosol of germ cells at all differentiative stages analysed with the exception of epididymal spermatozoa. Detergents are needed to solubilize the enzyme activity in spermatozoa, and further studies will be performed to establish whether the enzyme becomes particulate in this cell type. The calcium-calmodulin-independent phosphodiesterase with high affinity for cAMP shows chromatographic behaviour and kinetic properties similar to a germ cell-specific isoform described for the rat (Geremia *et al.*, 1982), and it can be distinguished from the hormone-dependent form of the somatic cells mainly because it elutes at lower salt concentration. Furthermore, it undergoes quantitative variations during spermatogenesis that are similar to those described for the rat (Geremia, Rossi, Conti & Stefanini, 1983b) and that correlate well with changes of intracellular cAMP levels during spermatogenesis (Adamo *et al.*, 1980a). The highest enzyme specific activity is found in early spermatids in which the lowest cAMP levels are detectable. This enzyme activity probably corresponds to the enzyme activity described in developing rat testis (Monn, Desautel & Christiansen, 1972) and in mature spermatozoa (Tash, 1976).

Even though it is not possible to speculate on the role of these enzyme activities in spermatogenesis, the finding that these phosphodiesterase forms are present in different species is of interest. Three activities are present in the rat (Purvis, Olsen & Hansson, 1981; Geremia *et al.*, 1982) and in the mouse (present study), i.e. the calcium-calmodulin-dependent form with high-affinity for cGMP (130 mM-salt) and the hormone-dependent form with high-affinity for cAMP in Sertoli cells (430 mM-salt), and the calcium-calmodulin-independent enzyme with high-affinity for cAMP in germ cells (350 mM-salt). In both species, the calcium-calmodulin independent enzyme shows similar quantitative variations during spermatogenesis. It is therefore tempting to speculate that these enzyme activities have a role in differentiative events of spermatogenesis. The fourth enzyme activity identified in mouse testis, i.e. the calcium-calmodulin-dependent form (110 mM salt) with high affinity for cAMP and cGMP, seems, on the contrary, to have a distribution that is species-specific. It is present and it represents a major form in germ cells of the mouse, while a similar form could not be identified in rat germ cells (Geremia *et al.*, 1982), adult rat testis (Purvis *et al.*, 1981) or testes from adult bulls, rabbits and trout (unpublished observations).

The two forms of phosphodiesterase identified in germ cells and specific to these cell types could represent genetic markers of spermatogenesis. Attempts are currently being made to purify germ cell-specific forms to establish whether they are gene products distinct from phosphodiesterase forms present in other tissues and cell types.

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