Testis-specific Calmodulin-dependent Phosphodiesterase

A DISTINCT HIGH AFFINITY cAMP ISOENZYME IMMUNOLOGICALLY RELATED TO BRAIN CALMODULIN-DEPENDENT cGMP PHOSPHODIESTERASE*

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A cell-specific isozyme of calmodulin (CaM)-dependent phosphodiesterase that exhibits micromolar affinity for cAMP has been purified 900-fold from mouse testis by DEAE chromatography, gel filtration, affinity chromatography with CaM-Sepharose 4B, and isoelectric focusing. The highly purified enzyme is stimulated 5-6-fold by CaM in the presence of Ca2+ and hydrolyzes both cAMP and cGMP with anomalous substrate dependence, i.e. high and low affinity components (Km 2 and 20 μM) are observed either in the presence or absence of CaM. Each of the substrates acts as a noncompetitive inhibitor of the other, suggesting the presence of two distinct catalytic sites on the enzyme. Hydrodynamic studies suggest that the testis phosphodiesterase is an asymmetric monomer of 68-70 kDa that forms a dimer after interaction with Ca2+ and CaM; the tetrameric complex exhibits an apparent molecular size of 180 kDa. These enzymatic and biophysical properties differ in many respects from those of the brain isozyme, suggesting that they are different proteins. Nevertheless, common epitopes do exist, since the testis enzyme interacted with rabbit antibodies raised against bovine brain CaM-dependent phosphodiesterase. The major peptide of 68 kDa was strongly reactive on immunoblots, and was distinguished unambiguously from the 60-kDa species from mouse brain. A comparison of the immunoreactive fragments produced by limited proteolysis with staphylococcal V-8 protease indicated several similarities in the domains of these polypeptides. Thus, although differing in several important physical and biochemical parameters, the testis enzyme appears immunologically related to CaM-dependent phosphodiesterase from brain. On the basis of these data, we conclude that common elements of the structural genes for these isozymes have been conserved, whereas certain biological properties, including substrate specificity, have diverged substantially.

The most extensively characterized forms of calmodulin (CaM)-dependent1 phosphodiesterase, purified to apparent homogeneity from bovine brain and heart using ion-exchange chromatography (1), CaM-Sepharose affinity chromatography (2-5), and conformation-specific monoclonal antibody chromatography (6), show high affinity (Km 2-4 μM) for cGMP and much lower affinity (Km >20 μM) for cAMP. Although quite similar in terms of substrate specificity, it now appears that these represent a family of tissue-specific forms of the enzyme. In addition to these, CaM-dependent phosphodiesterases with high affinity for cAMP or for both cyclic nucleotides have been described recently in liver membranes (7), immature rat testis (8), rat pancreas (9), male mouse germ cells (10), and a human lymphoblastoid cell line (11). Unfortunately, highly purified preparations of these have not been available for rigorous characterization.

We were interested in establishing whether the "high affinity cAMP-cGMP" CaM-dependent phosphodiesterases are enzymes distinct from the "low affinity cAMP" forms or whether they represent degraded or posttranslationally modified forms of the latter. To date, no conclusive answers to this question have been given (12). Nonetheless, in a study performed in male mouse germ cells (10), we suggested on the basis of kinetic and physicochemical data that the high affinity cAMP-cGMP CaM-dependent phosphodiesterase from mouse testis was different from the low affinity cAMP calmodulin-dependent phosphodiesterase that appears to be the predominant form of the enzyme in other tissues.

Here we report the purification of the enzyme from mouse testis and present biochemical evidence confirming that it is distinct from the CaM-dependent low affinity cAMP form. However, we also provide data indicating that the two enzymes share physical domains that are immunologically related, suggesting common elements in the structural genes for these isozymes.

EXPERIMENTAL PROCEDURES

Materials—Cyclic nucleotides (Sigma) and radiolabeled 3H-cyclic nucleotides (Du Pont-New England Nuclear, specific activity, 1 mCi/mmol) were routinely purified by gel filtration. Cyanogen bromide-activated Sepharose 4B was from Pharmacia LKB Biotechnology Inc. Protein standards for gel filtration, sucrose density analysis, and electrophoresis were from Boehringer Biochemicals. Snake venom nucleotidase (Ophiophagus hannah) was from Sigma. All other reagents, purchased from various sources, were of analytical grade.

1 The abbreviations used are: CaM, calmodulin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; EGTA, [ethylendiamine(oxycyclenitril)]-tetraacetic acid; Hapes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby indicated this fact.
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**Purification Procedure**—All purification steps were performed at 4°C. Approximately 100 testes from adult mice (strain CD1) were decapsulated, freeze-thawed, and homogenized in 3 volumes of 20 mM Tris-HCl (pH 7.2), 0.5 mM EGTA, 10 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride in an all-glass Dounce homogenizer, using 15 strokes with a loose pestle followed by 10 strokes with a tight pestle. The homogenate was centrifuged (30 min, 20,000 x g) and the resulting supernatant centrifuged at 100,000 x g for 1 h. The 100,000 x g supernatant, referred to as cytosol, was diluted 1:4 with 70 mM sodium acetate (pH 6.5), 0.2 mM EGTA, 10 mM 2-mercaptoethanol and loaded onto a DE52 (Whatman) column (bed volume, 30 ml) equilibrated with the same buffer. After washing with 3 column volumes, a linear gradient of 70–600 mM sodium acetate (pH 6.5) was started. Elution was performed at a flow rate of 1 ml/min and 3-ml fractions were collected. Fractions containing CaM-dependent phosphodiesterase activity were pooled and immediately concentrated by ultrafiltration (Amicon, FM-30 membrane, 40 psi).

The concentrated enzyme (1–2 ml) was applied to a Sephacryl S-200 column (V = 95 ml) equilibrated with 70 mM sodium acetate (pH 6.4), 2 mM CaCl2, 1 mM EGTA, 5 mM MgCl2, 10 mM 2-mercaptoethanol, and 1 mM NaN3. 1-ml fractions were collected at a flow rate of 4 ml/h. Fractions containing CaM-dependent phosphodiesterase activity were pooled and loaded onto such, or following desalting on Sephadex G-25 columns, onto a column of CaM-Sepharose 4B (bed volume, 5 ml) equilibrated with 25 mM Hepes (pH 7), 2 mM CaCl2, 1 mM EGTA, 5 mM MgCl2, 1 mM NaN3. The initial eluate was reapplied to the column, which was then washed with 15 ml of the equilibration buffer and 30 ml of the same buffer containing 0.4 M NaCl. The enzyme was eluted with 9 ml of 25 mM Hepes (pH 7), 5 mM EGTA, 5 mM MgCl2, 1 mM NaCl.

The recovered enzyme was immediately concentrated by diafiltration on Centricon-30 membranes (Amicon) and subjected to liquid phase isoelectric focusing in a reaginer apparatus (LKB model 3100). Focusing was performed in a sucrose-Ampholine step gradient (24 steps, 4.6 ml/step; 0–50% (w/v) sucrose; 1–3.5 (v/v) Ampholine (LKB), pH range 3.5–10). The concentration sample was included in the middle fraction of the step gradient. A constant voltage (400 V) was applied for 64 h. Fractions (1.86 ml) containing phosphodiesterase activity were pooled and ultrafiltered to remove Amipholines and to concentrate enzyme activity. Enzyme was stored at –20°C in 30% glycerol.

Aliquots from the various purification steps were concentrated by diafiltration and subjected to sodium 3-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 8% acrylamide gels. Fixed gels were stained by the silver nitrate method (13).

**Immunochromusical Procedures**—Antibodies against CaM-dependent bovine brain phosphodiesterase were raised in adult male rabbits (the Jackson Laboratory, strain AJX-1) and the IgG fraction was purified by protein A-Sepharose chromatography as described (14).

**Other Procedures**—CaM was routinely purified from bovine brain by using affinity chromatography with phenylthiazine derivatives coupled to Sepharose 4B essentially as described (15), or by melittin-Sepharose chromatography (17).

**RESULTS**

Purification of CaM-dependent High Affinity cAMP-cGMP Phosphodiesterase—DEAE-cellulose chromatography of cytosol from adult mouse testis was performed in the presence of protease inhibitors as described under "Experimental Procedures." The 100,000 x g supernatant was diluted 5-fold with starting buffer containing 0.2 mM EGTA and chromatographed on a column (0.7 x 9 cm) of DEAE-cellulose, as described under "Experimental Procedures." Phosphodiesterase activities toward 1 μM CAMP (upper panel) or 1 μM CgMP (lower panel) were assayed with (21) or without (22) 1 mM Ca2+ and purified calmodulin (3 μg/ml).

FIG. 1. DEAE-cellulose chromatography of cytosol from mouse germ cells.

Homogenates of isolated germ cells were prepared in the presence of protease inhibitors as described under "Experimental Procedures." The 100,000 x g supernatant was diluted 5-fold with starting buffer containing 0.2 mM EGTA and chromatographed on a column (0.7 x 9 cm) of DEAE-cellulose, as described under "Experimental Procedures." Phosphodiesterase activities toward 1 μM CAMP (upper panel) or 1 μM CgMP (lower panel) were assayed with (21) or without (22) 1 mM Ca2+ and purified calmodulin (3 μg/ml).

**FIG. 1.** DEAE-cellulose chromatography of cytosol from mouse germ cells. Homogenates of isolated germ cells were prepared in the presence of protease inhibitors as described under "Experimental Procedures." The 100,000 x g supernatant was diluted 5-fold with starting buffer containing 0.2 mM EGTA and chromatographed on a column (0.7 x 9 cm) of DEAE-cellulose, as described under "Experimental Procedures." Phosphodiesterase activities toward 1 μM CAMP (upper panel) or 1 μM CgMP (lower panel) were assayed with (21) or without (22) 1 mM Ca2+ and purified calmodulin (3 μg/ml).

RESULTS

Purification of CaM-dependent High Affinity cAMP-cGMP Phosphodiesterase—DEAE-cellulose chromatography of cytosol from adult mouse testis was performed in the presence of EGTA to dissociate the CaM-dependent enzyme from endogenous CaM and to obtain maximum binding of the enzyme to CaM-Sepharose in a subsequent affinity chromatography step. Enzyme activity eluted from the DEAE column in two main peaks (Fig. 1): a first peak, representing the CaM-dependent enzyme form previously characterized in male mouse germ cells (10), eluted at 110 mM sodium acetate, hydrolyzed micromolar concentrations of cAMP and CgMP equally well, and was activated about 3–4-fold by CaM2+; a second smaller peak, eluting at 350 mM sodium acetate, was specific for cAMP and was not stimulated by CaM. Overall, the CaM-dependent activity represented >90% of the phosphodiesterase activity present in the soluble fraction from this tissue when assayed with 1 μM CgMP; with 1 μM CAMP,

*The enzyme activity eluted as a symmetrical peak consistent with a globular protein of ~100 kDa, and retained its CaM sensitivity during this step. Furthermore, when fractions containing enzyme activity*
were pooled and loaded onto a CaM-Sepharose 4B affinity column, activity was quantitatively bound, indicating that inactivation or proteolysis of the preparation had not occurred. The affinity chromatography step typically gave a recovery of enzyme activity between 15 and 30%, and the highest degree of purification (30-fold) when compared to the other steps of the procedure. After concentration by ultracentrifugation, the enzyme was further fractionated by preparative isoelectric focusing. This step, which resulted in 100% recovery of enzyme activity, was meant initially to be analytical and was later utilized in the purification procedure because of the reproducibility high yields; the apparent stability of the activity may be, in part, due to the protective effect of the sucrose and/or Ampholines. In addition, a further 2-fold purification was achieved with the separation of trace amounts of a low affinity cAMP CaM-dependent phosphodiesterase which has a slightly lower isoelectric point. The testis-specific enzyme (isoelectric point, 5.6) retained hydrolytic properties and CaM sensitivity indistinguishable from those of less purified fractions. When the purified enzyme preparation was analyzed by SDS-PAGE, a single peptide of approximately 68 kDa was observed (Fig. 2, inset).

A typical purification, beginning with 100 mice, required about 3–4 days and gave a final yield of 5%, as shown in Table I, with a 900-fold enrichment; the specific activity of the preparation was 1.0 µmol/min/mg of protein when assayed with 1 µM cAMP. Enzyme activity was stable up to 5 months when stored at -20 °C in 30% glycerol.

**Enzymatic and Physical Characterization of the Purified Enzyme—**

![Enzymatic and Physical Characterization of the Purified Enzyme](image)

**Fig. 2.** Preparative isoelectric focusing of CaM-dependent phosphodiesterase from mouse testis. Isoelectric focusing of the CaM-Sepharose eluate was carried out as described under “Experimental Procedures”; enzyme activity was assayed in the absence (○) or in the presence (●) of CaM. Inset: SDS-PAGE of concentrated pooled fractions (PF) 23–29 (pI ~ 5.5–5.7); molecular mass standards are shown at left.

<table>
<thead>
<tr>
<th>Table I</th>
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<tr>
<td><strong>Purification of the CaM-dependent cAMP-cGMP phosphodiesterase from mouse testis</strong></td>
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<tr>
<td>Protein</td>
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</tr>
<tr>
<td>Cytosol</td>
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<tr>
<td>DEAE-cellulose</td>
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<td>Sephacryl S-200</td>
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<tr>
<td>CaM-Sepharose</td>
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<td>Isoelectric focusing</td>
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* Enzyme activity was assayed with 1 µM cAMP as substrate.
with CaM, the enzyme may associate to form a tetrameric αβ2 complex.

The presumed monomeric form of the enzyme also behaved anomalously on nondenaturing polyacrylamide gel electrophoresis (6% acrylamide) showing an Rf of 0.07, compared to a value of 0.23 shown by a CaM-dependent low affinity cAMP isoenzyme from somatic cells of the mouse testis (23) (Fig. 6). This electrophoretic behavior is consistent with the germ cell form being an elongated molecular form having greatly reduced mobility.

**Immunoochemical Characterization**—Polyclonal rabbit antibodies (IgG fraction), raised against purified bovine brain phosphodiesterase, were tested for cross-reactivity with both the mouse brain and mouse testis enzymes. These antibodies have been extensively characterized (14, 15) and shown to be specific for the CaM-dependent form of the enzyme. Western blot analysis of the mouse brain enzyme showed reactivity essentially equivalent to that seen for bovine, rat, or rabbit brain (data not shown), indicating interspecies similarities.

Western blotting of partially purified DEAE fractions, as well as the purified testis enzyme, showed a primary immunoreactive band of 68 kDa (Fig. 7, lane 1T), in agreement with the result obtained by silver staining (Fig. 2, inset); in parallel experiments, mouse brain enzyme showed an immunoreactive band of 69 kDa (Fig. 7, lane B) identical with the bovine brain enzyme (5). In some large preparations, during which it was difficult to prepare tissue rapidly, a series of apparent breakdown products were observed, suggesting that the enzyme was very susceptible to degradation by testicular proteases (data not shown). Interestingly, these degraded preparations were stimulated 2–3-fold by CaM and had been prepared by adsorption to CaM-Sepharose, indicating their ability to bind CaM. Since certain of the lower Mr, immunoreactive peptides from testis were never observed in preparations of brain enzyme, it suggested that either the phosphodiesterases or the proteases from the two tissues differed markedly.

To test whether the major immunoreactive peptides from mouse testis and brain were highly related, Western blotting of protease-treated enzymes were carried out, as shown in Fig. 7 (lanes 2 and 3). This analysis indicated that, although the pattern of proteolytic fragments of the two enzymes clearly differed substantially, a number of fragments (38, 33, 31, and 25 kDa) appeared to be shared by both enzymes. However, unique peptides of 27 and 28 kDa were generated only from testis enzyme.

**DISCUSSION**

A CaM-dependent phosphodiesterase with high affinity for cAMP and cGMP has been purified approximately 1000-fold from adult mouse testis. Although other CaM-dependent phosphodiesterase activities with high affinity for cAMP (7, 8) or both cAMP and cGMP (9–11) have been described, this

<table>
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<tr>
<th>SDS-PAGE peptide size (kDa)</th>
<th>Brain enzyme</th>
<th>Testis enzyme</th>
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<tbody>
<tr>
<td>60</td>
<td>+CaM</td>
<td>-CaM</td>
</tr>
<tr>
<td>48</td>
<td>-</td>
<td>40</td>
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<tr>
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<td>K&lt;sup&gt;a&lt;/sup&gt; cAMP (µM)</td>
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<tr>
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<td>0.3</td>
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<td>1.0</td>
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<td>Stimulation by CaM (fold)</td>
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<td>3–6</td>
</tr>
<tr>
<td>K&lt;sup&gt;a&lt;/sup&gt; cAMP vs cGMP (µM)</td>
<td>20 (competitive)</td>
<td>20 (non-competitive)</td>
</tr>
<tr>
<td>K&lt;sup&gt;a&lt;/sup&gt; cGMP vs cAMP (µM)</td>
<td>2 (competitive)</td>
<td>2 (non-competitive)</td>
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<tr>
<td>K&lt;sup&gt;a&lt;/sup&gt; spermine (µM)</td>
<td>0.1</td>
<td>0.2</td>
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* Most of the data reported for the bovine brain enzyme are from Ref. 5.
* Data from Ref. 4.
* Calculated from gel filtration and sucrose density gradient studies.
* Data from Ref. 23 (the low affinity cAMP enzyme from somatic cells of mouse testis).
Testis-specific Isozyme of CaM-dependent Phosphodiesterase

FIG. 4. CaM stimulation of the purified phosphodiesterase from mouse testis. Enzyme was assayed with 1 μM cAMP in the presence of 1 mM CaCl₂ and the indicated concentrations of purified CaM. Data represent mean ± S.D. of triplicate determinations.

FIG. 5. Determination of the Stokes radius of purified phosphodiesterase in the presence and absence of CaM. Concentrated enzyme (10 μg) from the isoelectric focusing step was fractionated on a column of Sephacryl S-200 (1.2 × 100 cm), equilibrated in buffer containing 1 mM EDTA (A), or Sephacryl S-300 column equilibrated in buffer containing 1 mM CaCl₂ (B). In B, the enzyme was mixed with excess CaM prior to chromatography. Enzyme activity was assayed in the absence (○) or presence (○) of CaM. bsa, bovine serum albumin; V₀, void volume; Vₑ, total bed volume.

FIG. 6. Nondenaturing electrophoresis of purified phosphodiesterases from mouse testis. Highly purified preparations of CaM-dependent phosphodiesterase from either somatic germ cells from immature mouse testis (panel A) or male mouse germ cells (panel B) were electrophoresed under nondenaturing conditions on a rectangular (slab) polyacrylamide gel (6% acrylamide). The lanes were cut into 25 3-mm sections, eluted with buffer, and assayed for phosphodiesterase activity in the presence of CaM as described in Ref. 17. The dye front was found in slice 30, hence Rₑ values of peak enzyme activity are determined relative to this mobility. Recovery of enzyme activities from gel slices was 55–60% of the total applied to the gel.

FIG. 7. Comparative immunoblots of calmodulin-dependent phosphodiesterase isoenzymes from mouse brain and testis. Purified CaM-dependent phosphodiesterases from mouse testis (lanes T) and brain (lanes B) were electrophoresed and transferred electrophoretically to nitrocellulose. Samples (2 μg/50 μl) had been incubated (30 min, 25 °C) with 0 (lanes 1), 4 (lanes 2), and 40 (lanes 3) μg/ml staphylococcal V-8 protease. After incubation with rabbit anti-phosphodiesterase, the blot was developed further with goat anti-rabbit conjugated to alkaline phosphatase (Promega Biotec) and visualized according to the manufacturer’s instructions.

The report provides the first characterization of the enzyme following extensive purification. The purified enzyme shows a subunit structure which is regulated physically as well as enzymatically by CaM in the presence of Ca²⁺. The molecular size in the absence of CaM was calculated to be 70 kDa, approximately the subunit molecular size of 68 kDa obtained both by SDS-PAGE and
phodiesterase, or high affinity cAMP phosphodiesterase) (15). We are comparing highly related but discrete molecules. The presence, albeit in different cell populations (10), and high affinity cAMP CaM-dependent phosphodiesterases tend our comparison to a low affinity cAMP CaM-dependent enzyme. The bovine brain enzyme shows high affinity for cAMP and cGMP as well as noncompetitive inhibition of one substrate by the other (data not shown). These reciprocal inhibition studies confirmed earlier observations with partially purified preparations (10) and strongly suggest that the enzyme possesses distinct catalytic sites for cAMP and cGMP. The maintenance of allosteric interactions either in the presence or absence of CaM, i.e. either in the monomeric or dimeric state of the enzyme, suggests that both sites are present on each subunit. This enzyme, representing the predominant form of male mouse germ cell phosphodiesterase (10), shows several features that distinguish it from the class of CaM-dependent phosphodiesterases that have been purified from brain and heart (1-5) and identified in several other tissues, including the somatic cell compartment of the testis (23) (for a review, see Ref. 12). In Table II, we compare the properties of the brain CaM-dependent enzyme (5) with those of the testis enzyme. The bovine brain enzyme shows high affinity for cGMP and low affinity for cAMP. This low affinity cAMP form is a dimeric enzyme composed of two 60-kDa subunits either in the presence or absence of CaM. In our hands, the brain and testis enzyme show the same Ks, even though a lower P value has been reported (4). Indeed, when compared directly in three separate experiments, we found that the enzymes from these different tissues gave precisely the same isoelectric point. Spermine inhibited basal activity of both enzymes with similar Ks, but the extent of inhibition was approximately 70% for the brain enzyme (22), but only about one-half that for the testis enzyme. Recently, we have extended our comparison to a low affinity cAMP CaM-dependent phosphodiesterase identified in the somatic cells of mouse testis (23). As seen with the brain isozyme, this enzyme shows competitive reciprocal inhibition between substrates, suggesting the presence of a single catalytic site. Moreover, the electrophoretic mobility (under non-denaturing conditions) of the low affinity cAMP form is essentially identical to that observed for the bovine brain enzyme (18). These observations strengthen the hypothesis that, in testis, distinct low affinity and high affinity cAMP CaM-dependent phosphodiesterases are present, albeit in different cell populations (10). The immunological studies with the antibodies to bovine brain CaM-dependent phosphodiesterase demonstrate that we are comparing highly related discrete molecules. The antigenicity is apparently not due to some general structural feature of phosphodiesterases, as the antibodies do not react with any of the other major classes of this enzyme (i.e. cyclic GMP-stimulated phosphodiesterase, rod outer segment phosphodiesterase, or high affinity cAMP phosphodiesterase) (15). Although the holoenzymes from testis germ cells and brain have different biophysical and biochemical properties, common epitopes must be present in both polypeptides to account for the high degree of cross-reactivity. Indeed, many of the immunoreactive peptides appear to be identical between the brain and testis isoforms, indicating substantial homologies in the major peptide domains. In comparing the pattern of peptides produced by treatment with V-8 protease, we found a high degree of similarity between that of the 60-kDa bovine brain isoform, published by Sharma et al. (24), and the major phosphodiesterase form isolated from murine brain. Since the latter form shares reactive epitopes with the 88-kDa peptide, it would appear that the germ cell enzyme is more closely related to the 60-kDa species than to the 63-kDa isoform from brain. The significance of this relatedness is not clear at present. The existence of closely related CaM-dependent isozymes of phosphodiesterases in testis and brain poses both teleological and functional questions. The different species may have arisen from some common ancestral gene or may be the consequence of alternative processing of a precursor mRNA, although the presence of two apparently distinct catalytic sites on the testis enzyme makes the latter possibility seem unlikely. At a biochemical level, it is intriguing that the primary difference appears to be an increased affinity for cAMP in the germ cell isoform. It is thought that cAMP metabolism plays an important role in germ cell maturation and in sperm cell mobility (for a review see Ref. 25) and germ cells are known to possess soluble as well as particulate adenylyl cyclase activities (26). It seems plausible that close regulation of cytosolic cAMP levels via phosphodiesterase may be required to control specific CaM-dependent functions in these cells. Interestingly, the somatic cells from testis contain essentially only the cGMP-specific isozyme, suggesting different priorities for cyclic nucleotide metabolism in these cell types. Since, in testis, different CaM-dependent phosphodiesterases are produced within adjacent cell populations, this tissue may provide an important model for examination of influences on cell-specific gene expression and/or messenger RNA regulation. Preparation of molecular probes for the brain enzyme should permit direct examination of these possible modes of regulation.

REFERENCES