Characterization of a calmodulin-dependent high-affinity cyclic AMP and cyclic GMP phosphodiesterase from male mouse germ cells

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Two cyclic nucleotide phosphodiesterase activities were separated by ion-exchange chromatography of cytosol from male mouse germ cells. A form eluted at low salt concentration showed high affinity ($K_m \approx 2 \mu\text{M}$) and low affinity ($K_m \approx 20 \mu\text{M}$) for cyclic AMP, and high affinity ($K_m \approx 3.5 \mu\text{M}$) for cyclic GMP. A second form, eluted at high salt concentration, showed high affinity ($K_m \approx 5 \mu\text{M}$) for cyclic AMP and was similar to a phosphodiesterase activity described in rat germ cells. The present study was performed to characterize the first form, which represents most of the phosphodiesterase activity in mouse germ cells. The enzyme was sensitive to Ca^{2+} and calmodulin stimulation, which increased its activity 3–4-fold. Calmodulin stimulation depended on direct interaction of the activator with the enzyme, as indicated by the reversible changes in the chromatographic elution pattern in the presence of Ca^{2+} , as well as by the increase in the sedimentation coefficient in the presence of calmodulin. Reciprocal inhibition kinetics between cyclic AMP and cyclic GMP for the calmodulin-dependent form demonstrated a non-competitive inhibition between the two substrates, suggesting the presence of separate catalytic sites. This is in agreement with kinetic parameters and different thermal stabilities of cyclic AMP- and cyclic GMP-hydrolysing activities. Furthermore, the relevant change in s value, depending on the absence or presence of Ca^{2+} and calmodulin, suggested that the enzyme is composed of subunits, which aggregate in the presence of the activator. A model for catalytic site composition and reciprocal interaction is also proposed.

The existence of cyclic nucleotide phosphodiesterase forms regulated by calmodulin is clearly established. The best-described form of calmodulin-dependent phosphodiesterase shows high affinity for cyclic GMP and low affinity for cyclic AMP. This form has been described in several tissues and species (reviewed by Wells & Hardman, 1977; Beavo *et al.*, 1982) and purified and extensively characterized in bovine heart and brain (Ho *et al.*, 1977; LaPorte *et al.*, 1979; Klee *et al.*, 1979; Sharma *et al.*, 1980). Similar enzymic forms have been described also in rat testis tissue (Means *et al.*, 1978; Purvis *et al.*, 1981; Geremia *et al.*, 1982).

Ca^{2+} -dependent phosphodiesterases with different substrate specificities have been described. A calmodulin-regulated activity with high affinity for cyclic AMP from hamster liver mem-

branes (Smoake *et al.*, 1981) was reported, and a similar activity was tentatively identified in bovine lung (Beavo *et al.*, 1982). A calmodulin-dependent enzyme with high affinity for cyclic AMP that also hydrolyses cyclic GMP with high affinity has furthermore been demonstrated in immature rat testis (Purvis *et al.*, 1981) and in chick heart (Andrenyak & Epstein, 1982), and a similar form has been purified from rat pancreas (Vandermeers *et al.*, 1983).

We report here on the identification in male mouse germ cells of a calmodulin-stimulated phosphodiesterase activity hydrolysing both cyclic AMP and cyclic GMP with high affinity and representing most of the cyclic nucleotide-hydrolytic activity in this cell type. The mechanism of calmodulin activation, the physicochemical and kinetic properties of this activity and the reciprocal inhibition between substrates were investigated. A possible model of interaction between sites is also discussed.

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Materials and methods

Germ-cell preparation

Germ-cell suspension was obtained from adult male Swiss CD1 mice. Decapsulated testes were incubated with 0.1% collagenase I (Boehringer) in Dulbecco's phosphate-buffered saline (0.9 mM- CaCl_2 / 2.7 mM- KCl / 1.47 mM- KH_2PO_4 / 0.49 mM- MgCl_2 / 137 mM- NaCl / 8.1 mM- Na_2HPO_4 , pH 7.4) at room temperature with continuous shaking (50 cycles/min) for 10–15 min until the seminiferous tubules were dispersed. The resulting seminiferous tubules were washed three times by sedimentation in phosphate-buffered saline for 10 min at unit gravity, and then cut into 2–4 mm fragments with a razor blade. After three washes with phosphate-buffered saline, fragments were digested with 0.1% trypsin ('trypsin 1:250'; Difco) and 0.01% deoxyribonuclease I (Boehringer) with continuous shaking for 30 min at 30°C. Digestion was terminated by adding bovine serum albumin fraction V (final concn. 0.1%). Undigested fragments were separated from released germ cells by sedimentation for 10 min at unit gravity. Germ cells in the supernatants were washed twice by centrifugation at 100g for 10 min in a clinical centrifuge.

Homogenate preparation

Germ-cell pellets containing about 200×10^6 cells were suspended in 3 ml of 20 mM-Tris/HCl (pH 8) / 10 mM-2-mercaptoethanol / 2 mM-phenylmethanesulphonyl fluoride / 0.2 mM-EGTA, unless otherwise stated in the Results section, and homogenized at 0°C with 15 strokes in an all-glass Dounce homogenizer. The homogenates were centrifuged at 20000g at 4°C for 20 min, and the resulting supernatants were centrifuged again at 105000g at 4°C for 1 h. The 105000g supernatant is referred to below as 'cytosol'. Protein content was determined by a modification of the method of Lowry (Bensadoun & Weinstein, 1976).

Phosphodiesterase assay

Enzymic activity was measured as conversion of cyclic AMP and cyclic GMP into their corresponding 5'-nucleotides, by the method of Thompson & Appleman (1971). The incubation mixture (final volume 200 μl) contained 20 mM-Tris/HCl, pH 8, 5 mM-2-mercaptoethanol, 0.1 mM-EGTA, 1 mM- CaCl_2 , 5 mM- MgCl_2 , and, when indicated, 3 μg of purified calmodulin/ml (Jamieson & Vanaman, 1979). Tritiated cyclic nucleotides (NEN; sp. radioactivity 36 Ci/mmol; 10^5 c.p.m./tube) were routinely purified by gel filtration. Cyclic AMP and cyclic GMP were used at a final concentration of 1 μM if not otherwise indicated in the Results section; enzyme concentration and time of reaction were adjusted so that no more than 20% of the substrate

was hydrolysed. The reaction performed at 34°C was started by adding cyclic nucleotide, and stopped by adding 10 mM-EDTA at 0°C and then boiling for 1 min. The second incubation, with *Crotalus atrox* venom (Sigma), and separation of the products by batch adsorption were as previously described (Thompson & Appleman, 1971).

Ion-exchange chromatography

DEAE-cellulose chromatography (DE52; Whatman) was performed as previously described (Geremia *et al.*, 1982). Cytosol containing 2–3 mg of protein was diluted 1:4 with equilibration buffer [70 mM-sodium acetate (pH 6.5) / 5 mM-2-mercaptoethanol / 0.2 mM-EGTA, unless otherwise indicated in the Results section] and applied to columns (0.7 cm $\times$ 9 cm). After washing with 4 column vol., elution was performed with 50 ml of a linear 70–600 mM-sodium acetate gradient (pH 6.5) containing 5 mM-2-mercaptoethanol and 0.2 mM-EGTA, unless otherwise indicated in the Results section, at a constant flow rate of 0.25 ml/min; 0.5 ml fractions were collected in tubes containing 50 μl of 0.1% bovine serum albumin.

Gel filtration

Samples (1.0 ml) of fractions from DEAE-cellulose chromatography containing the highest activity were directly applied to a column (1.0 cm $\times$ 110 cm) of Sephacryl S-200 (Pharmacia) equilibrated with 70 mM-sodium acetate (pH 6.5) / 10 mM-2-mercaptoethanol / 1 mM-EDTA. Elution was performed at 4°C with the same buffer at a flow rate of 3 ml/h (0.5 ml fractions were collected) with an hydrostatic pressure of 30 cmH₂O. The column was calibrated with Combithek calibration proteins II (Boehringer). $^3\text{H}_2\text{O}$ and ^{14}C -labelled bovine serum albumin (NEN) were used as standards to monitor migration.

Sucrose gradient analysis

Samples (0.3 ml) of fractions from DEAE-cellulose chromatography containing the highest activity were directly layered on 5–20% linear sucrose gradients. Buffer system, centrifugation conditions, M_r standards used and fraction collection were as previously described (Geremia *et al.*, 1982).

Polyacrylamide-gel electrophoresis

Buffer systems and electrophoresis conditions were those described by Kincaid & Vaughan (1979). Samples of fractions from DEAE-cellulose chromatography containing the highest activity were concentrated 6-fold by dialysis against dry Ficoll (Pharmacia) and equilibrated with reservoir buffer diluted 1:3 before electrophoresis. Gels were cut into 1.5 mm sections and placed into

100 μl of phosphodiesterase assay incubation mixture. After 24 h at 4°C, samples of the incubation mixture were assayed for enzyme activity (Marchmont & Houslay, 1980).

Results

Properties of germ-cell phosphodiesterases

Most of the phosphodiesterase activity of germ-cell homogenates was recovered in the soluble fraction. Subcellular-localization studies by sequential sedimentation showed that over 85% of the activity was recovered in the 105000g supernatant, the remaining activity being localized mainly in the 20000g pellet (10–12%), and only trace amounts (1–3%) could be detected in the 900g and 105000g pellets. The specific activity measured in the crude cytosol in the presence of Ca^{2+} and excess calmodulin was approx. 0.7 nmol of cyclic AMP and 0.5 nmol of cyclic GMP hydrolysed/min per mg of protein.

Two main peaks of hydrolytic activity, eluted at 110mM- and 350mM-sodium acetate respectively, were resolved by DEAE-cellulose chromatography of the cytosol prepared from germ cells (Fig. 1). The first peak hydrolysed both cyclic AMP and cyclic GMP, and was stimulated by Ca^{2+} and calmodulin, whereas the second peak hydrolysed only cyclic AMP and was Ca^{2+} - and calmodulin-independent. Recovery of hydrolytic activity was 80–

90% in all chromatographies performed. By integrating the area of the chromatographic peak, the calmodulin-dependent activity represented approx. 80% of the cyclic AMP-hydrolytic activity and almost 100% of the cyclic GMP-hydrolytic activity of the germ-cell cytosol, and the specific activity of this form was increased to approx. 5 nmol/min per mg of protein.

The Ca^{2+} -calmodulin-dependent cyclic AMP- and cyclic GMP-hydrolytic activity of the first peak was strictly dependent on the presence of Mg^{2+} in the incubation (results not shown). The hydrolysis of both cyclic AMP and cyclic GMP was maximal at pH 8 and was inhibited by 3-isobutylmethylxanthine, with an identical K_i of approx. 20 μM (results not shown). On the other hand, the two hydrolytic activities showed different sensitivity to temperature. Thermal-denaturation studies performed in the presence of excess bovine serum albumin (0.1%) at 50°C showed that the half-maximal inhibition of cyclic AMP hydrolysis occurred at 2 min and that of cyclic GMP hydrolysis at 5 min (Fig. 2).

Calmodulin stimulation of the cyclic nucleotide phosphodiesterase

In an attempt to distinguish between the properties of the cyclic AMP- and cyclic GMP-hydrolytic activities and to assess whether the two activities reside on different molecules, the mechanism of stimulation by Ca^{2+} and calmodulin was studied. Maximal enzyme activity was

Fig. 1. DEAE-cellulose chromatography of cytosol from mouse germ-cell suspension

Cytosol preparation and chromatography were performed in the presence of 0.2mM-EGTA. Cyclic AMP (a) and cyclic GMP (b) phosphodiesterase activities were assayed with (●) or without (■) 1mM- CaCl_2 and purified calmodulin (3 $\mu\text{g}/\text{ml}$).

Fig. 2. Effect of temperature on calmodulin-dependent phosphodiesterase activity

Samples (50 μl) from the DEAE-cellulose column were incubated at 50°C for different times in the presence of bovine serum albumin (1 mg/ml). Incubation was terminated by cooling in an ice bath, and cyclic AMP (●) and cyclic GMP (■) phosphodiesterase activities were measured in the presence of 1mM- CaCl_2 and purified calmodulin (3 $\mu\text{g}/\text{ml}$).

expressed only when both Ca^{2+} and calmodulin were present in the assay, and the presence of Ca^{2+} alone or the removal of Ca^{2+} by EGTA produced a marked decrease in the phosphodiesterase activity (results not shown). Again the stimulation of both cyclic AMP and cyclic GMP hydrolysis occurred at similar concentrations of purified calmodulin. In a manner similar to that shown for the cyclic GMP phosphodiesterase purified from bovine brain (Tucker *et al.*, 1981), a short trypsin treatment caused activation of hydrolysis of cyclic AMP and cyclic GMP with concomitant loss of calmodulin-sensitivity (Fig. 3), without any apparent modification of the elution of the enzyme from ion-exchange chromatography (results not shown). The reversal of the stimulation, complete in 15 min (Fig. 3), and the loss of enzymic activity observed after longer proteolytic treatment (results not shown), probably reflect hydrolysis of the catalytic site. Further evidence that the mechanism of calmodulin activation of the germ-cell phosphodiesterase is similar to the activation of the brain cyclic GMP phosphodiesterase came from the observation that the stimulation was sensitive to fluphenazine, which inhibited calmodulin stimulation with an apparent K_i of approx. $5\text{ }\mu\text{M}$ (results not shown); again, the hydrolysis of both nucleotides was equally sensitive to the phenothiazine inhibition.

The presence of Ca^{2+} during cytosol preparation and ion-exchange chromatography resulted in

Fig. 3. Effect of trypsin treatment on basal activity of the calmodulin-dependent phosphodiesterase

Samples ($50\text{ }\mu\text{l}$) from the DEAE-cellulose column were incubated at 34°C with or without trypsin ($50\text{ }\mu\text{g/ml}$) for different times. The incubation was terminated by adding 2-fold excess of soya-bean trypsin inhibitor and cooling at 0°C . Enzymic activity was measured in the presence of CaCl_2 after incubation with trypsin (●, cyclic AMP; ■, cyclic GMP), incubation without trypsin (○, cyclic AMP; □, cyclic GMP) and stimulation with calmodulin (▲, cyclic AMP; △, cyclic GMP). Each point is the mean of triplicates; s.d. was within 5% of the mean value.

modification of the elution properties of both hydrolytic activities, which were recovered at higher salt concentrations (250 mM-sodium acetate) as shown in Fig. 4(a). The modified chromatographic behaviour could be reversed by a second chromatography in the presence of EGTA (Fig. 4b); this is consistent with the hypothesis that calmodulin interacts with this enzyme in the presence of Ca^{2+} . However, neither of these procedures separated the cyclic AMP- and cyclic GMP-hydrolytic activities.

Sucrose-gradient analysis performed in the absence of calmodulin showed for both hydrolytic activities a peak at 4.5S (Fig. 5a), which was converted quantitatively into a 6.5S peak when excess calmodulin was added (Fig. 5b). This sedimentation behaviour differed from that shown by Ca^{2+} -dependent cyclic GMP-specific phosphodiesterase of the rat testis (Fig. 5c), which had s values of 6.8S and 7.7S depending on the absence or presence of calmodulin in the gradients.

Kinetic properties of the calmodulin-dependent phosphodiesterase

Most of the characteristics considered were similar for both cyclic AMP- and cyclic GMP-

Fig. 4. DEAE-cellulose chromatography of cytosol from mouse germ cells

Cytosol preparation and chromatography were performed in the presence of 1 mM- CaCl_2 (a). Fractions containing the enzymic activity eluted at 250 mM-sodium acetate were rechromatographed (b). Pooled fractions were adjusted to 70 mM-sodium acetate/2 mM-EGTA before rechromatography. Cyclic AMP (●)- and cyclic GMP (■)-hydrolytic activities were measured in the presence of 1 mM- CaCl_2 and purified calmodulin ($3\text{ }\mu\text{g/ml}$).

Fig. 5. Sucrose-gradient analysis of the calmodulin-dependent phosphodiesterase from mouse germ cells performed in the presence (a) or absence (b) of excess purified calmodulin

Cyclic AMP (●) and cyclic GMP (■) phosphodiesterase activity was assayed in the presence of 1 mM- CaCl_2 and 3 μg of purified calmodulin/ml. In (c) is reported, for comparison, the sedimentation behaviour of the calmodulin-dependent cyclic GMP-specific phosphodiesterase from rat germ cells centrifuged in the presence (▲) or absence (●) of calmodulin. In this case only cyclic GMP-hydrolysing activity was determined. In (a) the $s_{20,w}$ (○) values of standard proteins are shown. Standards used were: cytochrome c (1), ovalbumin (2), bovine serum albumin (3), aldolase (4) and catalase (5).

hydrolytic activities, except for thermal stability (Fig. 2), which indicated possible heterogeneity of the active sites. Lineweaver-Burk analysis of the enzymic activity at different substrate concentrations indicated that the calmodulin-dependent form has a high affinity for both substrates (Fig. 6). The kinetics for cyclic AMP hydrolysis were not linear, with apparent K_m values of approx. 2 μM and 20 μM , but a single K_m value of approx. 3.5 μM was found with cyclic GMP as substrate. The V_{max} values of the two high-affinity activities were in a ratio close to 1:1, and calmodulin stimulation increased V_{max} 3–4-fold for both substrates, without

Fig. 6. Lineweaver-Burk plots of substrate concentration curves of the calmodulin-dependent phosphodiesterase activity from DEAE-cellulose chromatography

The assay for cyclic AMP (a)- and cyclic GMP (b)-hydrolytic activities was performed in the presence of 1 mM- CaCl_2 and purified calmodulin (3 $\mu\text{g}/\text{ml}$) (■) or in the presence of 0.1 mM-EGTA (●). Each point is the mean of triplicates; s.d. was within 5% of the mean value.

affecting K_m values. If the total cyclic AMP-hydrolytic activity was considered (low- K_m and high- K_m activities) the cyclic AMP/cyclic GMP V_{max} ratio became 2:1.

Further evidence on the existence of distinct catalytic sites for the two substrates was obtained by studying the effect of different concentrations of the non-radioactive form of a nucleotide on three different concentrations of the labelled form of the other. Fig. 7 shows Dixon plots ($1/v$ versus $[I]$) of experiments performed in the presence (a, b) or absence (c, d) of Ca^{2+} and exogenous calmodulin. In both the presence and the absence of calmodulin there was a non-competitive inhibition by cyclic GMP of the cyclic AMP-hydrolysing activity (K_i approx. 3.5 μM) and by cyclic AMP of cyclic GMP-hydrolysing activity (K_i approx. 19 μM), indicating an allosteric interaction between sites. At higher inhibitor concentrations (25–100 μM ; not shown in Fig. 7) inhibition became competitive. These inhibition kinetics differed

Fig. 7. Effect of different concentrations of the non-radioactive form of a nucleotide on the hydrolysis of $0.3 \mu\text{M}$ (●), $0.6 \mu\text{M}$ (▲) and $1.2 \mu\text{M}$ (■) concentrations of the labelled form of the other (indicated in the panels)

The reciprocal of the enzymic activity measured in the presence (a and b) or absence (c and d) of 1 mM-CaCl_2 and purified calmodulin ($3 \mu\text{g/ml}$) is shown on the ordinate, by Dixon plots ($1/v$ versus $[I]$). Each point is the mean of triplicates; S.D. was within 10% of the mean value.

from those shown by Ca^{2+} -calmodulin-dependent cyclic GMP phosphodiesterase from rat testis (results not shown); in that case competitive inhibition between substrates was found at all cyclic nucleotide concentrations tested.

Separation of the cyclic AMP- and cyclic GMP-hydrolytic activities

Both DEAE-cellulose chromatography in the presence or absence of Ca^{2+} and sucrose-gradient centrifugation in the presence or absence of calmodulin had shown that the two hydrolytic activities migrated as a single peak. Similar results were obtained in at least two other separative systems. Co-elution of the two activities was observed when the first peak was analysed by Sephacryl S-200 gel filtration or by polyacrylamide-gel electrophoresis in non-denaturing conditions (results not shown). An apparent M_r of 65000 was calculated for both cyclic AMP and cyclic GMP forms. Further attempts to separate the two activities by ion-ex-

change chromatography after limited proteolytic digestion (Strada *et al.*, 1981) were ineffective. Thus the sites hydrolysing cyclic AMP and cyclic GMP could not be separated by at least these seven different analytical procedures.

Characteristics of the calmodulin-independent phosphodiesterase

The second peak of phosphodiesterase activity, eluted at 350 mM-sodium acetate, has not been extensively characterized in the present study. It represented approx. 20% of the cyclic AMP-hydrolytic activity recovered from the column and it probably corresponds to a form which has been previously described as germ-cell-specific in rat testis (Geremia *et al.*, 1982). The similarities between the two forms include chromatographic elution, Ca^{2+} - and calmodulin-insensitivity, specificity for cyclic AMP hydrolysis (Fig. 1) and a comparable K_m value, of approx. $5 \mu\text{M}$ (results not shown).

Discussion

Mouse male germ cells contain large amounts of a peculiar form of soluble phosphodiesterase activity with the following characteristics: (1) stimulation of the activity by Ca^{2+} and calmodulin; (2) high and low affinities for cyclic AMP and high affinity for cyclic GMP; (3) apparent allosteric interaction between cyclic GMP and cyclic AMP sites; (4) possible presence of both hydrolytic activities on a common subunit.

Full stimulation of enzyme activity was obtained when calmodulin was added to the assay together with Ca^{2+} , and the following evidence suggests a direct interaction of calmodulin with the enzyme. In the presence of Ca^{2+} , the sedimentation coefficient of enzyme alone was lower than that of the enzyme mixed with affinity-chromatography-purified calmodulin. In addition, the observation that chromatographic behaviour on DEAE-cellulose varied when the cation concentration was modified during homogenization and chromatography is consistent with the hypothesis of a reversible binding of the acidic calmodulin molecule to the enzyme. Similar changes of elution were observed for the calmodulin-dependent cyclic GMP-specific phosphodiesterase prepared from bovine heart (Ho *et al.*, 1977) and from rat testes (Geremia *et al.*, 1982). The calmodulin-dependent enzyme prepared from mouse germ cells was activated by limited proteolysis in a manner similar to that described for the cyclic GMP-specific calmodulin-dependent form (Tucker *et al.*, 1981); however, it is unlikely that this is the mechanism of calmodulin activation of the enzyme, since the Ca^{2+} -calmodulin-dependent activation was reversible, and proteolytic treatment of the enzyme did not cause any chromatographic change. Thus calmodulin interaction with the high-affinity cyclic AMP and cyclic GMP phosphodiesterase of the germ cells has the same characteristics as the brain cyclic GMP phosphodiesterase, including the sensitivity to phenothiazine.

On the basis of the kinetic data, three different catalytic sites could be distinguished: a low-affinity site for cyclic AMP (apparent $K_m \approx 20 \mu\text{M}$), a high-affinity site for cyclic AMP (apparent $K_m \approx 2 \mu\text{M}$) and a high-affinity site for cyclic GMP (apparent $K_m \approx 3.5 \mu\text{M}$). These findings could be explained by the co-elution in the same peak of a Ca^{2+} -dependent high-affinity cyclic AMP phosphodiesterase (Smoake *et al.*, 1981) and the Ca^{2+} -dependent cyclic GMP phosphodiesterase similar to that described in the brain, heart (reviewed by Beavo *et al.*, 1982) and rat testis (Geremia *et al.*, 1982). The last form is known to possess a high-affinity cyclic GMP phosphodiesterase and a low-affinity cyclic AMP phosphodiesterase activity. The different thermal stability of cyclic AMP- and cyclic GMP-hydrolytic activities also supports the

existence of distinct catalytic sites. However, the presence of two different enzymes is in contrast with the following observations: (a) the co-elution of the two activities in at least seven different separative procedures and (b) the presence of allosteric (non-competitive) interactions between substrates. Reciprocal-inhibition studies showed non-competitive inhibition kinetics both for cyclic AMP (apparent $K_i \approx 20 \mu\text{M}$) and for cyclic GMP (apparent $K_i \approx 3.5 \mu\text{M}$). Taking into consideration the kinetic data and the identity of the K_m and K_i for cyclic GMP ($3.5 \mu\text{M}$) and for cyclic AMP ($20 \mu\text{M}$), the simplest model that can be proposed is that of an enzyme possessing the following sites: a high-affinity cyclic AMP site, which is non-competitively regulated by cyclic GMP; a high-affinity cyclic GMP site, which acts as a catalytic and allosteric site, which is in turn non-competitively inhibited by cyclic AMP; a low-affinity catalytic cyclic AMP site, which non-competitively inhibits cyclic GMP hydrolysis. The low-affinity cyclic AMP site cannot be the same one that hydrolyses cyclic GMP; if this were the case, one would expect a competitive rather than a non-competitive inhibition by cyclic AMP of cyclic GMP hydrolysis. A competitive inhibition by cyclic AMP of cyclic GMP hydrolysis was observed for rat testis cyclic GMP-specific Ca^{2+} -dependent enzyme. These findings strengthen our hypothesis that the germ-cell calmodulin-dependent phosphodiesterase with high affinity for both cyclic AMP and cyclic GMP is different from the calmodulin-dependent cyclic GMP phosphodiesterase (reviewed by Beavo *et al.*, 1982).

The results of sucrose-gradient analysis, showing a large increase in s value after interaction with calmodulin, suggest that the enzyme is actually formed by subunits which associate in the presence of calmodulin. Since similar reciprocal-inhibition kinetics were observed in either the presence or the absence of the activator, and since none of the analytical techniques used allowed separation of hydrolytic activity for cyclic AMP from that for cyclic GMP, it can be hypothesized that the three catalytic sites described coexist in the same subunit, which could dimerize in the presence of the activator.

The enzymic activity that we have identified is not readily comparable with previously described forms from different tissues. This might be due, at least in part, to the fact that not all of the parameters that we have analysed have been described for other enzymic activities. Soluble forms of Ca^{2+} -calmodulin-dependent phosphodiesterase with high affinity for both cyclic AMP and cyclic GMP have been described in immature rat testis (Purvis *et al.*, 1981), in developing chick heart (Andrenyak & Epstein, 1982) and in rat pancreas (Vandermeers *et al.*, 1983); on the other hand, these forms had different sedimentation behaviour

or M_r values from the form that we have characterized, and reciprocal-inhibition studies to assess the kind of interaction between substrates were not reported. A soluble high-affinity cyclic AMP and cyclic GMP phosphodiesterase has also been described in rat uterus (Strada *et al.*, 1981); this form showed non-competitive inhibition between substrates, which was interpreted as the presence of distinct catalytic sites for cyclic AMP and cyclic GMP, but K_i values differed from K_m values and calmodulin-dependence was not considered. Furthermore, unlike the mouse germ-cell form, the cyclic AMP catalytic site of the rat uterus phosphodiesterase could be separated from the cyclic GMP catalytic site by limited proteolysis followed by chromatography.

In a previous study on phosphodiesterase from germ cells of the rat (Geremia *et al.*, 1982), no calmodulin-dependent high-affinity cyclic AMP activity was identified; on the other hand a calmodulin-independent phosphodiesterase was present showing similar chromatographic and kinetic properties to the Ca^{2+} -independent form of the mouse germ cells (Fig. 1). This enzymic activity was present in comparable amount (50–100 pmol of cyclic nucleotide hydrolysed/min per mg of protein loaded on the column) in both species. This calmodulin-insensitive enzyme probably belongs to the class of cyclic AMP-specific phosphodiesterases, which have been described in various other tissues (reviewed by Beavo *et al.*, 1982). Our data therefore suggest that the Ca^{2+} -independent catabolism of cyclic AMP in germ cells is regulated in the rat and in the mouse by a similar enzymic form. Conversely, striking quantitative and qualitative differences are observed in the Ca^{2+} -regulated catabolism of both cyclic nucleotides. The meaning of these differences cannot be explained at the moment. As for the possible functional significance of the reciprocal non-competitive inhibitory control between cyclic AMP and cyclic GMP, it must be emphasized that the K_i of each cyclic nucleotide is higher than the physiological intracellular concentrations (Spruill & Steiner, 1976; Adamo *et al.*, 1980) observed in the testis and in germ cells; therefore it could be a mechanism operating in extreme conditions.

Mouse germ-cell calmodulin-dependent phosphodiesterase is present in large amount in the soluble extract of these cells (0.7 nmol of cyclic nucleotide hydrolysed/min per mg of protein), and a single purification step by DEAE-cellulose chromatography produced a 10-fold purification (specific activity measured with $1 \mu\text{M}$ -cyclic AMP was 5–10 nmol hydrolysed/min per mg of protein). This specific activity is comparable with that of the similar form purified approx. 3000-fold from rat pancreas (Vandermeers *et al.*, 1983). Thus mouse germ cells represent an optimal source of

this form of phosphodiesterase for enzyme purification. An extensive purification will be required to obtain further data on the structural characteristics and functional significance of this Ca^{2+} -calmodulin-dependent high-affinity cyclic AMP and cyclic GMP phosphodiesterase of male mouse germ cells.

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