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CYCLIC AMP SPECIFIC, CALCIUM INDEPENDENT PHOSPHODIESTERASE FROM A MALIGNANT MURINE MAST CELL TUMOR

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SUMMARY

A cAMP specific phosphodiesterase (PDE) has been identified in a malignant tumor (P815) of murine mast cells. The PDE is found primarily (85%) in the soluble fraction of the cell. This enzyme, purified approximately 10-fold by gel filtration, occurs in a single molecular and kinetic form (low K_m), and is apparently not dependent on calcium and calmodulin for optimum activity. Although cGMP is hydrolyzed at only 4% of the rate of cAMP hydrolysis, this cyclic nucleotide inhibits cAMP PDE activity by 50-60% at a concentration of 25 μM.

INTRODUCTION

Multiple forms of cyclic nucleotide phosphodiesterase (PDE) have been identified in extracts of many mammalian tissues (1,2). These forms differ in their kinetic behavior, substrate specificity and response to various effectors and seem to exist in different ratios in various cell types. At least one form of these phosphodiesterases can be activated by calmodulin (3,4), an endogenous thermostable protein. Interaction of this enzyme with calmodulin and calcium results in a decrease in the K_m of the enzyme for substrate and an increase in V_{max}(3,4). However, a complete definition of the elements involved in the regulation of these various forms has not been achieved.

Intracellular concentrations of cyclic nucleotides are thought to influence cell proliferation and maturation. Furthermore, the cAMP content of some malignant tumors is lower than the cAMP levels in the corresponding normal tissues (5,6) and the in vivo administration of cAMP or theophylline causes retardation of tumor growth (7-9). Taken together, these data suggest that the proliferation of some malignant tumors is associated with lowered cAMP levels which can be explained, at least in part, by increased activity of the

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cAMP degradative enzyme PDE. With these views in mind, a study was undertaken to evaluate the nature of cyclic nucleotide phosphodiesterases in malignant murine mast cells. In this report we present evidence for a single activated (low K_m) form of phosphodiesterase which is calcium independent and which acts specifically with cyclic AMP as its substrate.

MATERIALS AND METHODS

Male Balb/c x DBA/F, hybrid mice (20g) were obtained from Charles River Laboratories. Neoplastic (P815) murine mast cells (10) were harvested and transplanted as previously described (11,12). [^{3}H]cAMP (Specific radioactivity, 39.8 Ci/mmol) and [^{3}H]cGMP (Specific radioactivity, 8.28 Ci/mmol) were purchased from New England Nuclear Corporation (Boston, MA). cAMP, cGMP and dithiothreitol were purchased from Calbiochem (San Diego, C.A.). Snake venom (Ophiophagus hannah) from Sigma Chemical Company (St. Louis, MO) was used as a source of 5'nucleotidase.

Murine mast cells were thawed and added to an equivalent volume of 0.05 M Tris-HCl, pH 7.4 and ultrasonicated (three 15 seconds intervals at 4°C with a 1 min. interval between each sonication), with a micro-ultrasonic cell disruptor. The cell sonicates were centrifuged at 40,000 x g for 20 min. at 4°C and the supernatant fraction was used for further studies. Phosphodiesterase activity was measured by the isotopic method of Filburn and Karn (13). Protein was estimated spectrophotometrically at 280 nm, by the method of Lowry et al. (14), or by use of the Biorad protein assay kit.

RESULTS

Substrate Specificity of Phosphodiesterase: The 40,000 x g supernatant of murine mast cell was tested for PDE activity with cAMP and cGMP as substrates. At the concentration of cyclic nucleotides (6.25 μM) presently used, the specific activity of cAMP PDE (5.41 nmoles/mg of protein 10 min) was found to be approximately 10-15 times higher than similarly purified PDE from other sources (e.g. bovine brain and bovine pineal). When the concentration of cAMP in the assay was varied from 1.25 - 62.5 μM, the apparent K_m for this substrate was 4 μM. No enzyme activity could be detected with cGMP as substrate at a concentration of 6.25 μM. However, when the cGMP concentration in the assay system was lowered to 1.0 μM, a small amount of cGMP hydrolysis (specific activity 0.21 nmoles/mg of protein) could be seen (data not shown). After repeated freeze-thawing, the majority (about 85%) of the PDE activity for cAMP was found in the soluble fraction of the cell. The enzyme activity (12-15%) remaining in the particulate fraction could be released by sonication. The particulate fraction also had little if any cGMP hydrolyzing activity.

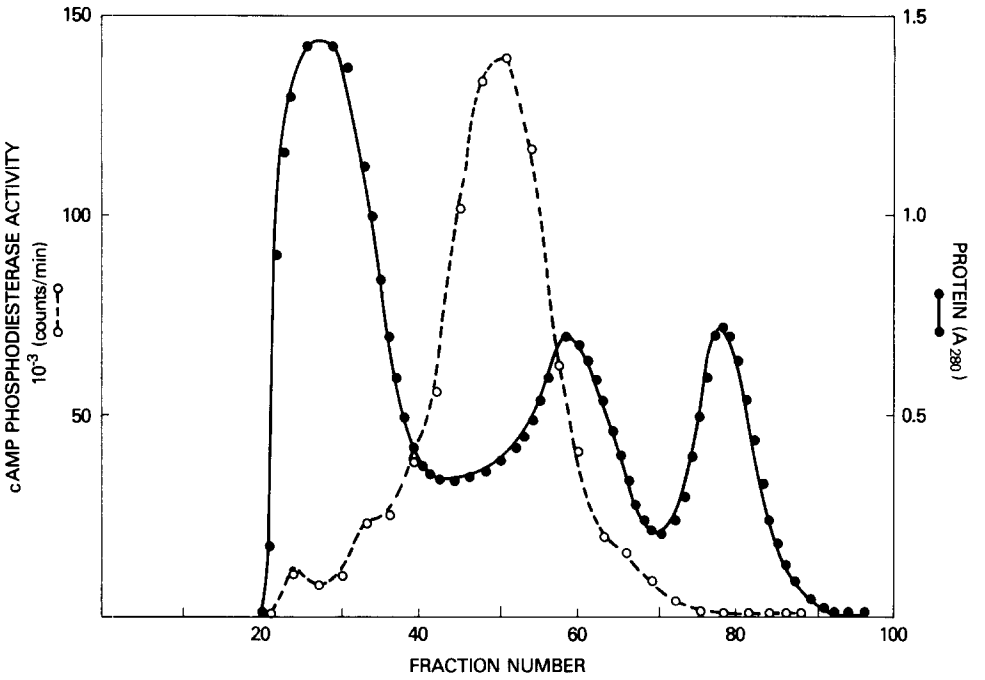

Figure 1 Elution profile of cAMP phosphodiesterase from murine mast cell on Sepharose 4B column. The 40,000 x g supernatant fraction (120 mg in 7 ml) was applied to a column (2.5 x 82 cm) of Sepharose 4B. Elution was carried out with 50 mM Tris-HCl buffer (pH 7.4) at a linear flow rate of 1.95 ml/cm^2-hr and 80 drop (5 ml) fractions were collected. Protein concentration was measured spectrophotometrically at 280 nm, and aliquots of each fraction were assayed for phosphodiesterase activity with cAMP and cGMP as substrates. No enzyme activity was observed with cGMP as substrate and hence, the data are not shown in this figure.

Single Molecular Form of Phosphodiesterase in Murine Mast Cell: When the 40,000 g supernatant fraction was chromatographed on a Sepharose 4B column (Fig. 1) the fractions possessing the enzyme activity with cAMP emerged as a single peak. No enzyme activity could be seen with cGMP in any of the fractions. As can be seen from the figure, the enzyme protein was resolved from the major protein peaks, eluting near the position where ferritin emerged from the column under similar conditions. The gel filtration step produced an approximate 10-fold increase in specific activity. This semi-purified preparation was used for all other studies.

TABLE 1

EFFECT OF CALMODULIN AND OTHER REACTION CONSTITUENTS ON MURINE MAST CELL PHOSPHODIESTERASE

Addition	cAMP converted p moles/10 min.
Enzyme	153.7
Enzyme + Calmodulin (1 μg)	155.4
+ EDTA (1 mM)	0
+ EGTA (1 mM)	154.9
- Ca^{++}	148.6
- DTT	163.3

Partially purified phosphodiesterase containing 4 μg of protein was employed in the assay system. Other reaction conditions are as described in the "Materials and Methods" section.

Effect of calmodulin on phosphodiesterase: The effects of various reaction constituents together with calmodulin are shown in Table 1. Like most other cyclic nucleotide PDEs, mast cell PDE has an absolute requirement for divalent Mg^{++} ions for enzyme activity. Dithiothreitol or Ca^{++} had no effect on the enzyme activity. The addition of calmodulin[1] to the assay system did not stimulate the enzyme activity either in the presence or absence of Ca^{++}. EGTA at a concentration of 1 mM did not inhibit the basal activity of the enzyme.

Effects of cGMP: The effects of varying concentrations of cGMP on the hydrolysis of cAMP by mast cell PDE are shown in Figure 2. At lower concentrations of cGMP (1-10 μM), the inhibition of enzyme activity was 25-40%. The inhibition of cGMP at a concentration of 25 μM was 56-60% and remained the same even at concentrations of cGMP up to 100 μM. As expected, aminophylline, theophylline, and caffeine, at concentrations of 5 mM each, produced 90-100% inhibition of PDE activity (data not shown).

[1] Highly purified calmodulin was a generous gift from Dr. I. Hanbauer

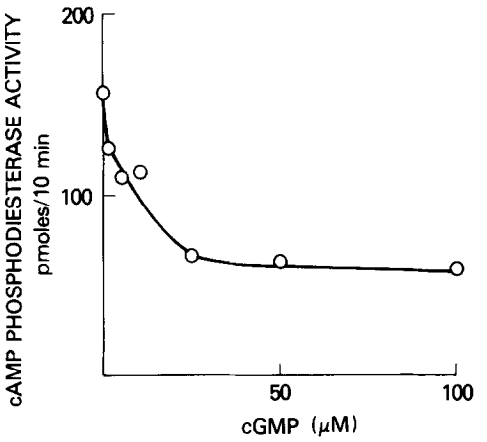

Figure 2 Effect of cGMP on the hydrolysis of cAMP by murine mast cell phosphodiesterase. Varying concentrations of cGMP were added to the assay system prior to the addition of ^{3}H cAMP. Enzyme aliquots from Sepharose 4B chromatography containing 6 μg of protein was employed in these studies. The concentration of cAMP in the assay system was 6.25 μM.

DISCUSSION

The foregoing results clearly indicate that the PDE in the malignant murine mast cell is a novel enzyme in many regards when compared to other phosphodiesterases from a variety of tissues (1,2). The enzyme is present primarily in the soluble fraction of the cell and emerges from Sepharose 4B as a single peak of activity. In fact, we have never observed more than one peak of enzyme activity in mast cell extracts purified by gel filtration (using, in addition to Sepharose 4B, Ultragel AcA22, Sephadex G-150, and Sephacryl S-300), sucrose density gradient centrifugation, ion exchange on DEAE cellulose, hydrophobic interaction chromatography, and isoelectric focusing (data to be presented elsewhere). In addition to existing as a single molecular form, the mast cell PDE is substrate specific for cAMP and it displays a singular kinetic (low K_m) form for this substrate. An apparent K_m for cAMP of 4 μM classifies this enzyme as a low K_m or "high affinity" PDE (1,2). In many regards the mast cell PDE described herein is similar to the cAMP phosphodeisterase in human monocytes described by Thompson et al. (15). The monocyte PDE also has a single molecular form ($s_{20,w}$=3.65, MW=45,000 determined by sedimentation only), a

single, high affinity kinetic form (1.3 μM for cAMP), and low cGMP hydrolyzing activity (15). In contrast to these similarities the murine mast cell PDE apparently does not resemble the PDEs from normal rat mast cell which have multiple kinetic and molecular forms (16).

Basal enzyme activity was not dependent on Ca^{++} and the addition of calmodulin with or without Ca^{++} did not stimulate activity. Similarly, the activity of a cAMP PDE from leukemic murine lymphocytes is not altered by either calmodulin or EGTA (17). It is known that calmodulin is tightly bound to phosphorylase kinase (18) and we cannot rule out the possibility at this time that calmodulin is also tightly bound to the mast cell PDE. However, enzyme purified through a DEAE ion exchange step, which usually results in a calmodulin free PDE (1,19), was, likewise, unresponsible to calmodulin and Ca^{++} (data not shown).

Earlier studies from our laboratory on bovine pineal phosphodiesterases showed that the cAMP hydrolysis of one form of PDE, which was acting on both cAMP and cGMP, was inhibited by μM concentrations of cGMP (20). Similar to the bovine pineal enzyme, the murine mast cell enzyme was also inhibited by cGMP, though the latter enzyme was hydrolyzing cAMP alone.

It has been suggested that malignancy is often associated with low effective levels of cAMP (5,6). The cytosol fractions of hepatoma cells are deficient in cAMP binding sites (21,22) and a shift in the elution profile of liver protein kinases, the activities of which were associated with cAMP, were reported in Yoshida ascites tumor bearing rats (23). The high relative specific activity of PDE, resulting in low levels of cAMP (preliminary experiments revealed that the concentration of cAMP in cell extracts is 5.3 nM and the concentration of cGMP is 5.2 nM), may therefore have some bearing on various aspects of altered cellular metabolism in the malignant mast cells.

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