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EVIDENCE FOR CONVERTIBLE FORMS OF SOLUBLE UTERINE CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

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The cyclic nucleotide phosphodiesterase (3′ : 5′-cyclic nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) systems of many tissues show multiple physical and kinetic forms. In contrast, the soluble rat uterine phosphodiesterase exists as a single enzyme form with non-linear Lineweaver-Burk kinetics for cyclic AMP (app. K_m of approx. 3 and 20 μM) and linear kinetics for cyclic GMP (app. K_m of approx. 3 μM) since the two hydrolytic activities are not separated by a variety of techniques. In uterine cytosolic fractions, cyclic AMP is a non-competitive inhibitor of cyclic GMP hydrolysis (K_i approx. 32 μM). Also, cyclic GMP is a non-competitive inhibitor of cyclic AMP hydrolysis (K_i approx 16 μM) at low cyclic GMP/cyclic AMP substrate ratios. However, cyclic GMP acts as a competitive inhibitor of cyclic AMP phosphodiesterase (K_i approx 34 μM) at high cyclic GMP/cyclic AMP substrate ratios. When a single hydrolytic form of uterine phosphodiesterase, separated initially by DEAE anion-exchange chromatography, is treated with trypsin (0.5 μg/ml for 2 min) and rechromatographed on DEAE-Sephacel, two major forms of phosphodiesterase are revealed. One form elutes at 0.3 M $NaOAc^-$ and displays anomalous kinetics for cyclic AMP hydrolysis (app. K_m of 2 and 20 μM) and linear kinetics for cyclic GMP (app. K_m approx. 5 μM), kinetic profiles which are similar to those of the uterine cytosolic preparations. A second form of phosphodiesterase elutes at 0.6 M $NaOAc^-$ and displays a higher apparent affinity for cyclic AMP (app. K_m approx. 1.5 μ) without appreciable cyclic GMP hydrolytic activity. These data provide kinetic and structural evidence that uterine phosphodiesterase contains distinct catalytic sites for cyclic AMP and cyclic GMP. Moreover, they provide further documentation that the multiple forms of cyclic nucleotide phosphodiesterase in mammalian tissues may be conversions from a single enzyme species.

Introduction

Cyclic nucleotide phosphodiesterase (3′ : 5′-cyclic nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) catalyzes the hydrolysis of cyclic nucleotides, the only known mechanism for catabolism of these regulatory compounds. Since the initial discovery of this enzyme system [1], many subsequent studies have established the existence of multiple forms with characteristic structural properties, kinetic behavior and sensitivities to inhibitors and activators [2–7]. Cyclic nucleotides themselves have also been shown to affect enzyme activity [6,8,9] and therefore are potential in vivo regulators of their own catabolism. A thorough understanding of each of these elements is essential to elucidate the role of phosphodiesterase in the regulation of cellular growth and function.

In contrast to phosphodiesterases from most mammalian sources [2–7], soluble rat uterine phosphodiesterase appears to exist as a single enzyme form which catalyzes the hydrolysis of both cyclic

Abbreviations: Bes, 2-(bis(2-hydroxyethyl)amino)ethanesulfonic acid; BHK, baby hamster kidney.

AMP and cyclic GMP [10]. Furthermore, estradiol treatment in vivo causes identical decreases in the activities of cyclic AMP and cyclic GMP phosphodiesterase [10].

Since uterine phosphodiesterase appears to exist as a single form [10] the kinetic studies reported here were undertaken to determine if separate catalytic sites exist for cyclic AMP and cyclic GMP, and to examine the effect of each cyclic nucleotide on the hydrolysis of the other. These studies are of interest because both cyclic AMP [11,12] and cyclic GMP [13,14] may play a role in the regulation of uterine growth and function.

The results of these kinetic studies suggest that the uterine phosphodiesterase contains separate catalytic sites for cyclic AMP and cyclic GMP hydrolysis. In conjunction with the kinetic experiments, limited proteolysis was used to determine if other enzyme forms which catalyzed the hydrolysis of only a single cyclic nucleotide could be generated from the homologous enzyme species. An enzyme form which had a high affinity for cyclic AMP and was devoid of cyclic GMP phosphodiesterase activity was in fact obtained after limited trypsin treatment. This additional form could be distinguished from the original native enzyme species by sucrose gradient centrifugation or DEAE-cellulose chromatography.

Materials and Methods

Materials. Cyclic[8-^{3}H]AMP (specific activity 16–23 Ci/mmol) and cyclic[8-^{3}H]GMP (specific activity 4.1 Ci/mmol) were purchased from Schwarz/Mann (Orangeburg, NY) and New England Nuclear (Boston, MA). [^{3}H]cyclic nucleotides were purified by Dowex 1-X8 200–400 mesh (BioRad Laboratories) anion-exchange chromatography and stored at −20°C in acidic-50% ethanol. Snake (*Ophiophagus hannah*) venom, cyclic AMP, cyclic GMP and ATP were obtained from Sigma (St. Louis, MO). All other reagents were the highest grade commercially available.

Preparation of tissue. Female Sprague-Dawley rats (20–22-day-old) were obtained from Texas Inbred Mice Co., Houston, TX. Rats were killed by decapitation and the uteri were removed and stripped of adherent fat and mesentery and placed in 40 mM Tris-HCl/0.32 M sucrose (pH 8.0). Uteri were homogenized in the above buffer (1 uterus/ml) with a tight-fitted Duall type glass homogenizer and centrifuged at 105 000 × *g* for 1 h. Using these procedures uterine cytosol preparations contain over 95% of the cyclic AMP and cyclic GMP phosphodiesterase activities present in the homogenate. Protein was measured by the method of Schacterle and Pollack [15] using bovine serum albumin as a standard.

Cyclic nucleotide phosphodiesterase assay. Cyclic AMP phosphodiesterase activity was measured by the radioisotope method of Thompson and Appleman [2]. The assay mixture (0.4 ml) contained 5 mM $MgCl_2$/40 mM Bes/5 mM β-mercaptoethanol/cyclic AMP (containing approx. 150 000 cpm cyclic[^{3}H]-AMP)/0.1 ml uterine cytosol (approx. 30–40 μg protein). Reactions were initiated by the addition of enzyme, incubated for 15 min at 30°C and terminated by boiling for 45 s. The [8-^{3}H]-adenosine was measured after treatment of the reaction mixture with snake venom (0.1 mg/ml) for 10 min to convert the 5′-AMP product and precipitation of unreacted substrate with anion-exchange resin as described previously [2]. Assays were performed at the indicated cyclic AMP substrate concentrations. Reactions were linear with respect to time and protein content. As reported by Rutten et al. [16] some recently purchased anion-exchange resin batches may non-specifically bind appreciable amounts of the adenosine. To minimize this problem, a resin slurry containing 40% methanol was used as described by Thompson et al. [17]. The blank values obtained with this procedure do not exceed those in the absence of methanol and product recoveries were 95% or greater.

Cyclic GMP phosphodiesterase activity was measured by the radioisotope procedure as described above, except that incubations were carried out for 10 min. Activity was measured with the indicated cyclic GMP substrate concentrations (containing approx. 130 000 cpm cyclic[^{3}H]GMP).

Results

Kinetic properties of uterine cyclic nucleotide phosphodiesterase

Fig. 1 shows previously published double-reciprocal plots [18] of uterine cytosolic cyclic nucleotide phosphodiesterase activities. Extrapolation

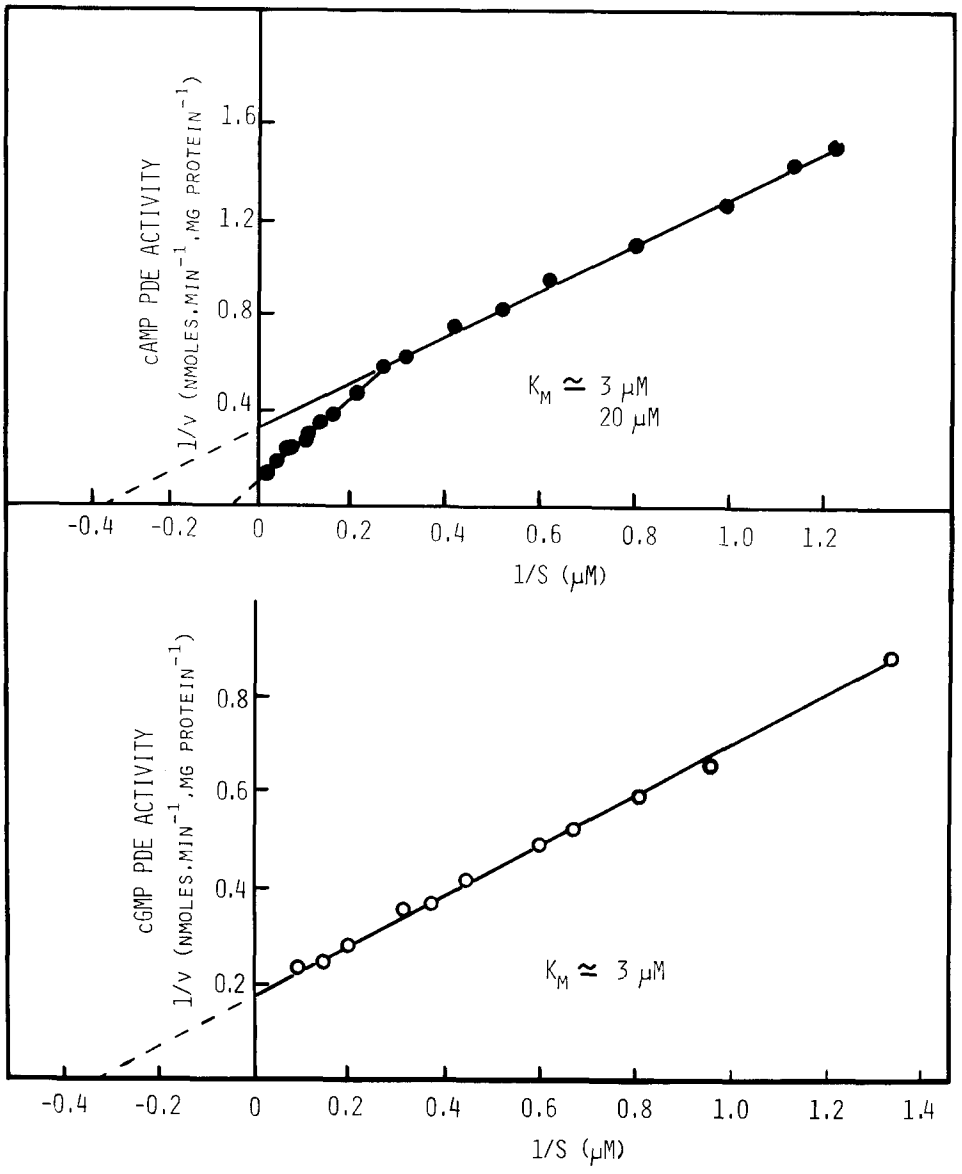

Fig. 1. Kinetic analysis of uterine cyclic nucleotide phosphodiesterase activity. Representative Lineweaver-Burk plot of uterine cytosol cyclic AMP phosphodiesterase (PDE) (upper panel) and cyclic GMP phosphodiesterase (lower panel) activities measured. V values are 3 and 10 nmol/min^{-1} (mg protein)$^{-1}$ for the low and high K_m cyclic AMP activities, respectively, and 5.6 nmol/min^{-1} (mg protein)$^{-1}$ for cyclic GMP phosphodiesterase. These data have been published previously [10] but are shown here as a reference for the present kinetic studies.

of the data for cyclic AMP phosphodiesterase (Fig. 1, upper panel) yields apparent K_m values of 3 and 20 μM. Maximum velocities of 3 and 10 nmol/min per mg protein are obtained for the low and high K_m activities, respectively. In contrast cyclic GMP phosphodiesterase (Fig. 1, lower panel) exhibits linear Lineweaver-Burk plots with an apparent K_m of 3 μM and a maximum velocity of 5.6 nmol/min per mg protein.

Dixon [19] plots illustrate the activity of cyclic GMP phosphodiesterase measured at different substrate levels in the presence of varying concentrations

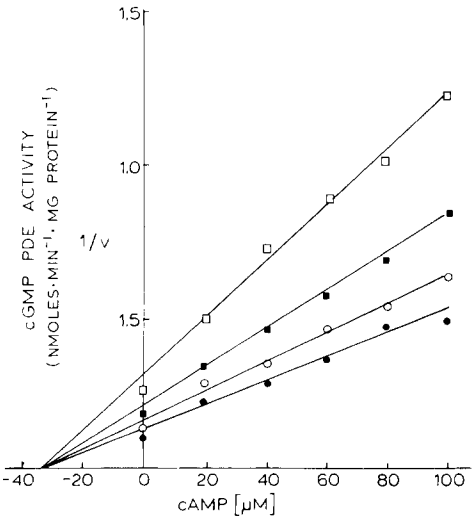

Fig. 2. Inhibition of cyclic GMP phosphodiesterase by cyclic AMP. Cyclic GMP phosphodiesterase (PDE) activity was measured at substrate concentrations of 0.4–1.0 μM in the presence of the indicated concentrations of cyclic AMP. Data are plotted according to Dixon [19] and a K_i of 34 μM cyclic AMP was determined graphically. Concentrations of cyclic GMP: □———□, 0.4 μM; ■———■, 0.6 μM; ○———○, 0.8 μM; ●———●, 1.0 μM.

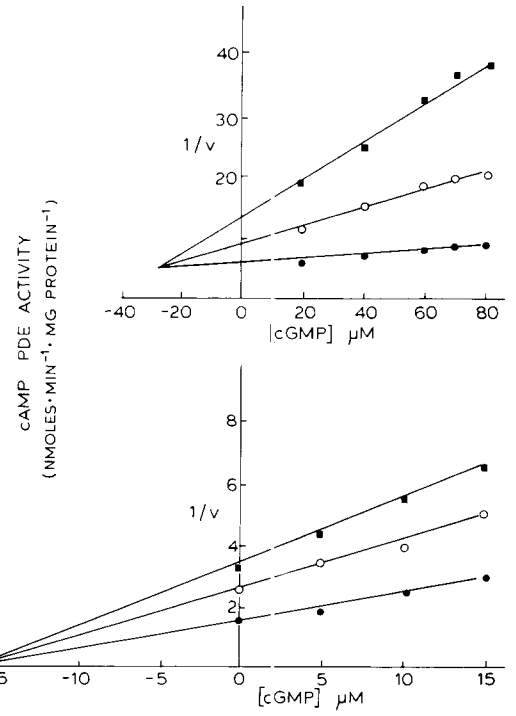

Fig. 3. Inhibition of cyclic AMP phosphodiesterase by cyclic GMP. Cyclic AMP phosphodiesterase (PDE) activity was measured at substrate concentrations of 0.2–1.0 μM in the presence of high (upper panel) and low (lower panel) concentration ranges of cyclic GMP. K_i values of 30 μM cyclic GMP (upper panel) or 16 μM cyclic GMP (lower panel) were observed for the competitive and non-competitive inhibition, respectively, determined from Dixon plots [19]. Concentrations of cyclic AMP, upper panel: ■———■, 0.2 μM; ○———○, 0.4 μM; ●———●, 1.0 μM and lower panel: ■———■, 0.4 μM; ○———○, 0.6 μM; ●———●, 1.0 μM.

of cyclic AMP (Fig. 2). Cyclic AMP appears to function as a non-competitive inhibitor (K_i approx. 34 μM) of cyclic GMP hydrolysis. Initial analysis of cyclic AMP phosphodiesterase activity determined in the presence of cyclic GMP yielded non-linear Dixon plots over a wide range of cyclic GMP concentrations. We, therefore, studied this inhibition at both low and high ratios of cyclic GMP/cyclic AMP since the nature of the inhibition appeared qualitatively different in the two concentration ranges. The data in the lower panel of Fig. 3 indicate that cyclic GMP is a non-competitive inhibitor of cyclic AMP phosphodiesterase at low cyclic GMP/cyclic AMP ratios (K_i approx. 16 μM). In contrast, cyclic GMP competitively inhibits its cyclic AMP phosphodiesterase activity at higher cyclic GMP/cyclic AMP ratios (Fig. 3, upper panel). The K_i for this competitive type of inhibition ranged between 30 and 35 μM in several experiments.

Other studies have demonstrated that low concentrations of cyclic GMP can activate cyclic AMP phosphodiesterase from some mammalian tissues [6,8,9]. However, uterine cyclic AMP phosphodiesterase measured at substrate concentrations of 0.025 to 250 μM is not activated by cyclic GMP concentrations between 0.01 and 5 μM (unpublished data).

Proteolysis of uterine phosphodiesterase

The observation that cyclic AMP is a non-competitive inhibitor of cyclic GMP hydrolysis and vice versa suggested that distinct catalytic sites are present for catalyzing the hydrolysis of the two cyclic nucleotides. To test this hypothesis proteolysis was used as a method to obtain enzyme forms specific for hydrolysis of only one of the cyclic nucleotides. Brief incubation with low levels of trypsin (<1 μg/ml) actually increased cyclic AMP phosphodiesterase activity (Fig. 4). The time course of activation with 0.5 μg/ml trypsin (Fig. 5) shows a peak effect at

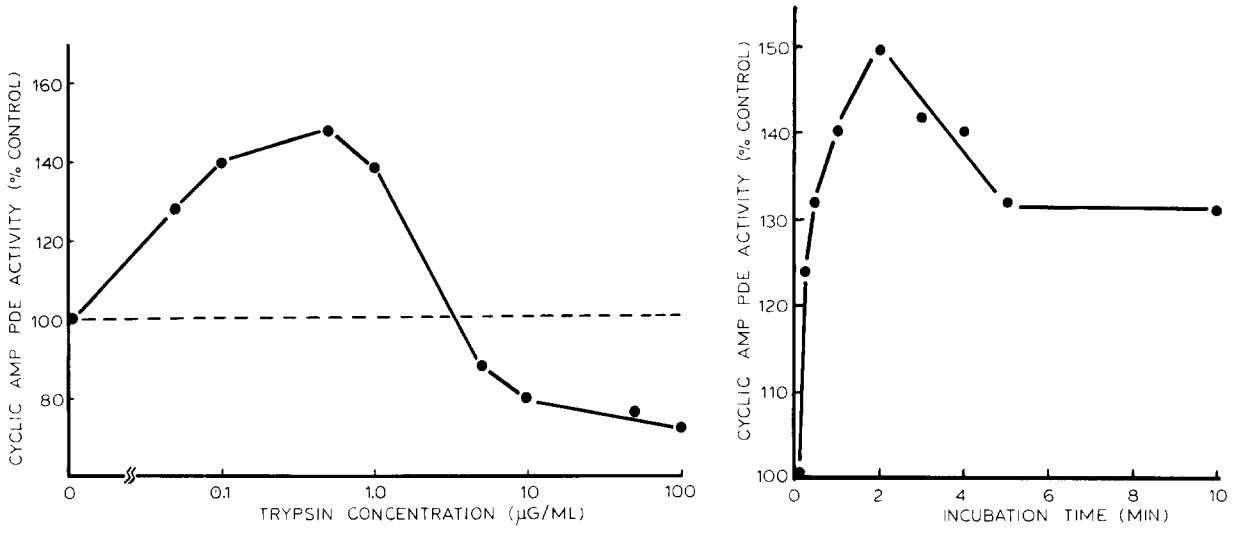

Fig. 4. Effect of trypsin concentration on cyclic AMP phosphodiesterase activity. Uterine cytosol was incubated under the conditions shown for 5 min at pH 8 and 30°C, and a 2-fold excess of lima bean trypsin inhibitor added prior to assay with 0.25 μM cyclic AMP substrate.

Fig. 5. Time course of trypsin activation of cyclic AMP phosphodiesterase activity. See legend to Fig. 4. 0.5 μg/ml trypsin was incubated at pH 8, and 30°C.

2 min. Using these treatment conditions (i.e., 0.5 μg/ml trypsin for 2 min) we next determined the nature of the enzyme forms present after proteolysis.

We have previously shown that a single species (7 S) which catalyzes the hydrolysis of cyclic GMP and cyclic AMP at both high and low substrate concentrations is resolved when untreated uterine cytosol is sedimented through a 5–20% sucrose gradient. The kinetic profiles of the pooled 7 S region of the gradient (unpublished data) are indistinguishable from those of the untreated cytosol profile shown in Fig. 1. However, when uterine cytosol is treated with trypsin before application to the gradient, an additional peak of cyclic AMP phosphodiesterase, devoid of appreciable cyclic GMP phosphodiesterase activity, is observed at 4 S [30]. When cyclic AMP phosphodiesterase activity is measured at a higher substrate level, two peaks of activity are observed at 3.5–4 S and 5.5–6 S and cyclic GMP phosphodiesterase activity appears to co-migrate only with the 6 S species [30].

We have also shown previously that a single form of phosphodiesterase activity which catalyses both cyclic AMP and cyclic GMP hydrolysis is observed when uterine cytosol is chromatgraphed on DEAE-cellulose [10]. Thus, in these studies we examined the effect of proteolysis on the structural properties of uterine phosphodiesterase with anion-exchange chromatography. Uterine cytosol was chromatographed on DEAE-Sephacel and the single enzyme form, which eluted at 0.3 M sodium acetate, obtained as reported previously [10]. This material (approx. a 10-fold purification of phosphodiesterase activity) was then treated briefly with trypsin and rechromatographed on DEAE. The results of this study (Fig. 6) show two peaks of activity eluting at 0.3 and 0.6 M salt. If the material separated in the initial chromatography step is rechromatographed, a single enzyme form eluting at 0.3 M salt was obtained (unpublished data). It is clear from the profile shown in Fig. 6 that peak II (which elutes at 0.6 M salt) is relatively specific for cyclic AMP hydrolysis while peak I contains both cyclic AMP and cyclic GMP hydrolytic activities.

The kinetic profiles for both cyclic AMP and cyclic GMP hydrolysis of the DEAE-separated peak I (Fig. 7) are essentially the same as those seen in Fig. 1 for the untreated cytosolic fraction. On the

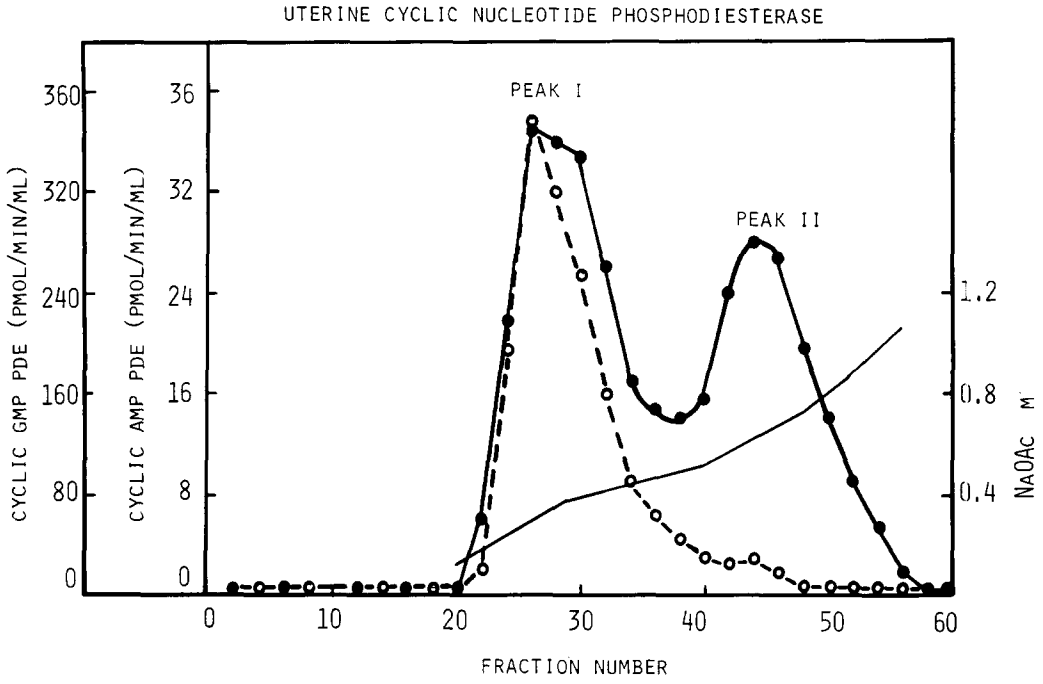

Fig. 6. DEAE-Sephacel chromatography of uterine cyclic nucleotide phosphodiesterase activity. Uterine phosphodiesterase was separated by chromatography of cytosol. The purified material was treated with trypsin (0.5 μg/ml for 2 min at 30°C), a 2-fold excess of trypsin inhibitor added, and the sample rechromatographed on DEAE-Sephacel as shown. Cyclic GMP activity (broken line) was assayed with 1 μM substrate and cyclic AMP activity (solid line) with 0.25 μM substrate after elution with a sodium acetate gradient as illustrated. Column recoveries of the applied cyclic AMP (•) and cyclic GMP phosphodiesterase (○) activities were approx. 75%.

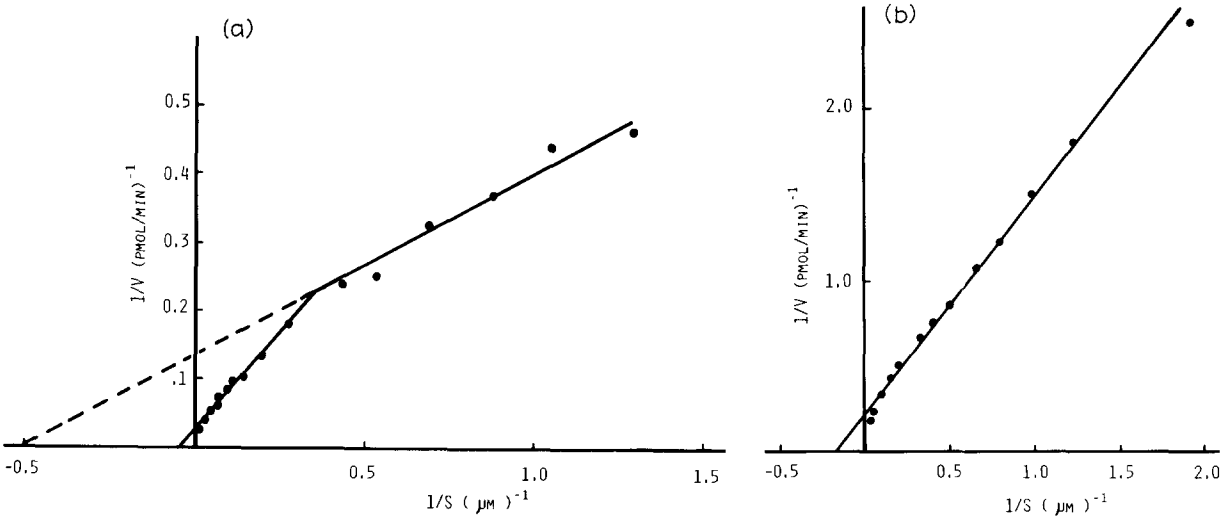

Fig. 7. Kinetics of trypsin-treated cyclic nucleotide phosphodiesterase after DEAE-chromatography. Pooled peak I fractions (0.3 M salt) (Fig. 6) were assayed with the indicated concentrations of substrates a, cyclic AMP phosphodiesterase K_m approx. 2 and 20 μM: b, cyclic GMP phosphodiesterase K_m approx. 5 μM.

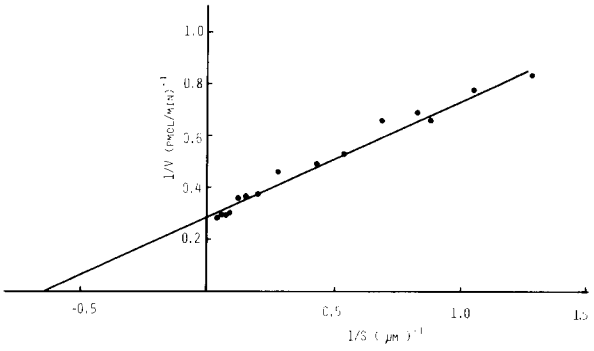

Fig. 8. Kinetics of trypsin-treated cyclic nucleotide phosphodiesterase. Pooled peak II fractions (0.6 M salt) (Fig. 6) were assayed with 0.25 μM cyclic AMP substrate. Cyclic AMP phosphodiesterase K_m approx. 1.5 μM.

other hand, the cyclic AMP phosphodiesterase activity present in the DEAE-separated peak II exhibits linear Lineweaver-Burk kinetics over a wide range of substrate concentrations with no evidence of a high K_m activity component (Fig. 8). Similarly, kinetic analysis of the lower molecular weight 4 S enzyme form generated by trypsin treatment of the uterine cytosol reveals only a low K_m enzyme form of cyclic AMP phosphodiesterase activity with no evidence of the "higher K_m" component (data not shown).

Discussion

Uterine cyclic nucleotide phosphodiesterase exhibits kinetic profiles similar to those reported for phosphodiesterase from other tissues. The soluble cyclic AMP phosphodiesterase displays non-linear Lineweaver-Burk plots with extrapolated apparent K_m values of 3 and 20 μM (Fig. 1). d'Auriac and Meyer [20] previously observed values of 2 and 36 μM for soluble uterine phosphodiesterase from diethylstilbesterol-treated immature rats. The uterine enzyme values are similar to those reported for hepatic phosphodiesterase [2,3], but lower than values reported for brain [2,21] and soluble rat kidney phosphodiesterases [22]. In contrast, cyclic GMP phosphodiesterase exhibits linear Lineweaver-Burk kinetics with a K_m of 3 μM (Fig. 1). The present studies utilized 20–22-day-old immature rats and differing values may be dependent upon animal age [23] as well as tissue, species, and procedural variables [22].

Kinetic analyses of the effects of cyclic nucleotides on enzyme activity indicate that uterine phosphodiesterases contain separate catalytic sites for cyclic AMP and cyclic GMP, since each substrate is a non-competitive inhibitor of the hydrolysis of the other. At higher concentrations cyclic GMP is also a competitive inhibitor of cyclic AMP phosphodiesterase, which is perhaps not unexpected based on the structural similarities between the two cyclic nucleotides. The observation that the apparent K_i values for the non-competitive inhibition are considerably higher than the respective K_m values suggests that uterine phosphodiesterase contains at least two sites for interactions with each cyclic nucleotide: a catalytic site for substrate hydrolysis and an allosteric site which affects the hydrolysis of the other substrate.

It is not clear at present what role, if any, these putative allosteric sites play in regulating phosphodiesterase activity in vivo. Basal uterine levels of cyclic AMP and cyclic GMP are approx. 1.4 [24] and 0.5 μM [25], respectively. While the apparent K_i values for inhibition of phosphodiesterase are considerably higher than these values, cellular concentrations of both cyclic nucleotides can be greatly increased in the uterus by appropriate stimuli [11,13,14,24]. A role for cyclic nucleotides in regulating uterine phosphodiesterase in vivo should therefore be considered a possibility in certain physiological situations.

Numerous studies with phosphodiesterases from different sources have demonstrated a variety of effects of cyclic nucleotides themselves on hydrolytic activity. In a number of cases, cyclic nucleotides have different effects on multiple enzyme forms present in the same tissue [27–29]. Despite the large number of effects observed in other systems, alterations in soluble uterine phosphodiesterase activity produced by cyclic nucleotides appear to represent a unique set of effects. In this regard, uterine phosphodiesterase most closely resembles a soluble form of rat liver phosphodiesterase described by Russell et al. [28] and a soluble form of bovine heart phosphodiesterase described by Ho et al. [29]. In the case of the liver enzyme each cyclic nucleotide non-competitively inhibits the hydrolysis of the other, but the apparent

K_i values are the same as the respective K_m values [28]. In the heart system each cyclic nucleotide competitively inhibits the hydrolysis of the other, however, the apparent K_i for cyclic GMP is approximately an order of magnitude greater than the apparent K_m for cyclic GMP hydrolysis [29]. It seems likely therefore, that soluble uterine phosphodiesterase has unique kinetic properties which may result from the occurrence in this tissue of a homologous enzyme species which catalyzes the hydrolysis of both cyclic nucleotide substrates [10]. The kinetic studies reported here support the conclusion that soluble uterine phosphodiesterase exists as a single enzyme species with separate catalytic sites for cyclic AMP and cyclic GMP hydrolysis. Direct support for this model is provided by physical criteria [30] and by the use of limited proteolysis of a single uterine enzyme form to achieve form conversion (this study).

Previous studies in rat uterus have demonstrated that a low K_m, cyclic AMP specific, low molecular weight (3.6–4.0 S) enzyme form can be derived from a less specific, single higher molecular weight (7 S) form upon activation by trypsin. In BHK cells, we have shown that the activity of a 6.7 S form for cyclic AMP is increased by storage at 4°C, treatment with trypsin, or treatment with rat brain or BHK fibroblast activator (calmodulin) proteins. Moreover, linear sucrose gradient fractionation has demonstrated that the activation of this enzyme peak by trypsin but not by calmodulin leads to the formation of a 3.6 S cyclic AMP specific form. We have now shown that limited proteolysis of a single DEAE-separable soluble uterine form which elutes at low salt concentration leads to the appearance of a low K_m relatively specific cyclic AMP form which elutes at a salt concentration higher than does the form isolated from the untreated soluble fraction. It is interesting to note the striking similarity between the '3.6–4 S form' and the 'high salt' form of the uterine enzyme produced by proteolysis and a high affinity form of cyclic AMP phosphodiesterase recently purified from dog kidney without exogenous trypsin treatment [31]. Each of these forms is relatively specific for cyclic AMP substrate, exhibits high affinity (low micromolar range), and lack activatibility by trypsin or calmodulin. Dog kidney also contains a form of cyclic nucleotide phosphodiesterase which elutes on DEAE-anion exchange chromatography at a salt concentration lower (peak I) than that which elutes the high affinity cyclic AMP specific form of phosphodiesterase (peak II). We have recently found that brief incubation of peak I from dog kidney at 37°C for 2 min results in the appearance of a cyclic AMP form of phosphodiesterase which will elute at high salt concentration upon rechromatography. In contrast as observed here with the uterine enzyme, the kidney cyclic GMP phosphodiesterase activity of the incubated peak I elutes on DEAE rechromatography at the same low salt concentrations as does the non-incubated peak I sample (unpublished data).

In an analogous series of experiments using a soluble fraction of coronary pig arteries Keravis et al. [33] resolved two peaks of phosphodiesterase activity (6.6 and 3.6 S) by sucrose gradient centrifugation. With regard to their substrate specificity and their sensitivity to calmodulin, these two sucrose gradient peaks were similar to two coronary phosphodiesterase activity peaks separated by DEAE-cellulose chromatography. In fact these two forms share many of the properties of forms isolated from dog kidney [31], cultured BHK cells [32], or rat uterine enzyme which had been subjected to limited proteolysis (Fig. 6). In contrast to our results and others using mouse lymphoma cells [34], the studies by Keravis et al. [33] failed to provide any evidence for the sensitivity of either of the two major coronary artery forms to trypsin or 'aging' of homogenates for 10 days at 4°C.

Recent evidence indicates that the activities of higher and lower molecular weight enzyme forms are influenced by proteins [35–37] in addition to calmodulin and trypsin. The cofacor dependency of these phosphodiesterase protein effectors or their modulation by cellular proteases is not known. Studies are in progress to test the influence of these effectors on purified enzymes to determine if they account for some discrepancies with partially purified enzyme preparations from different tissues.

Acknowledgements

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