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CATALYTIC AND REGULATORY PROPERTIES OF TWO FORMS OF BOVINE HEART CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

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Summary

The soluble supernatant fraction of bovine heart homogenates may be fractionated on a DEAE cellulose column into two cyclic nucleotide phosphodiesterases (EC 3.1.4.-): P_I and P_{II} phosphodiesterases, in the order of emergence from the column. In the presence of free Ca^{2+}, the P_I enzyme may be activated several fold by the protein activator which was discovered by Cheung ((1971) J. Biol. Chem. 246, 2859—2869). The P_{II} enzyme is refractory to this activator, and is not inhibited by the Ca^{2+} chelating agent, ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetate (EGTA). The activated activity of P_I phosphodiesterase may be further stimulated by imidazole or NH_4^+, and inhibited by high concentrations of Mg^{2+}. These reagents have no significant effect on either the P_{II} enzyme or the basal activity of P_I phosphodiesterase. Although both forms of phosphodiesterase can hydrolyze either cyclic AMP or cyclic GMP, they exhibit different relative affinities towards these two cyclic nucleotides. The P_I enzyme appears to have much higher affinities toward cyclic GMP than cyclic AMP. K_m values for cyclic AMP and cyclic GMP are respectively 1.7 and 0.33 mM for the non-activated P_I phosphodiesterase; and 0.2 and 0.007 mM for the activated enzyme. Each cyclic nucleotide acts as a competitive inhibitor for the other with K_i values similar to the respective K_m values. In contrast with P_I phosphodiesterase, P_{II} phosphodiesterase exhibits similar affinity toward cyclic AMP and cyclic GMP. The apparent K_m values of cyclic AMP and cyclic GMP for the P_{II} enzyme are approx. 0.05 and 0.03 mM, respectively. The kinetic plot with respect to cyclic GMP shows positive cooperativity. Each cyclic nucleotide acts as a non-competitive inhibitor for the other nucleotide. These kinetic properties of P_I and P_{II} phosphodiesterase of bovine heart are very similar to those of rat liver cyclic GMP and high K_m cyclic AMP phosphodiesterases, respectively (Russel, Terasaki and Appleman, (1973) J. Biol. Chem. 248, 1334).

Abbreviation: EGTA, ethyleneglycol *bis* (β-aminoethylether)-*N,N'*-tetraacetic acid.

Introduction

Multiple forms of cyclic nucleotide phosphodiesterase (EC 3.1.4.-) have been demonstrated in mammalian tissues by many investigators using a variety of approaches [1—12]. Beavo et al. [13] have suggested the presence of more than one form of the bovine heart cyclic nucleotide phosphodiesterase on the basis of kinetic properties and subcellular distribution of the enzyme. Subsequently, Goren and Rosen [14], and independently Hrapchak and Rasmussen [15] have fractionated the bovine heart enzyme into two fractions which have different kinetic properties. Preliminary results from our laboratory have also demonstrated the separation of two bovine heart cyclic nucleotide phosphodiesterases; a Ca^{2+}-activated and a Ca^{2+}-independent form [16,17]. The activation of the former enzyme depends on the presence of a protein activator [18] which was originally discovered by Cheung [19] in bovine brain. This protein activator has been purified to homogeneity [20] and identified as a Ca^{2+} binding protein [21]. Prior to our study, Kakiuchi et al. [12] demonstrated the separation of a Ca^{2+}-activated and a Ca^{2+}-independent cyclic nucleotide phosphodiesterase in rat brain.

In the present study, the kinetic and regulatory properties of the two forms of bovine heart enzyme are delineated. Portions of this work have been reported in meetings [16,17].

Materials and Methods

Cyclic AMP, cyclic GMP and snake venom 5′-nucleotidase were from Sigma. Cyclic [^{3}H]AMP and Cyclic [^{3}H]GMP were obtained from Schwarz-Mann. Anion-exchange resin AG1-X2 and DEAE cellulose, Cellex D were from Bio Rad. 2,5 Diphenyloxazole and 1,4-*bis*-2-(5-phenyloxazolyl)-benzene were purchased from Fisher. Bovine heart protein activator was prepared according to a procedure previously described [20].

Phosphodiesterase preparations. The two forms of bovine heart cyclic nucleotide phosphodiesterase were prepared according to the modification of a procedure previously used for the preparation of the activator-deficient cyclic nucleotide phosphodiesterase [18]. Frozen bovine heart slices (400 g) were thawed and homogenized in 2.5 vols. of a buffer, pH 7.5, containing 20 mM Tris and 1 mM EDTA. The homogenate was centrifuged at 1500 rev./min for 30 min, the supernatant collected, and its pH adjusted to 8.0. This supernatant was then brought to 60% saturation of $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$ and centrifuged at 8500 rev./min for 20 min. The pellet was suspended in 20 mM Tris/1 mM imidazole/10 μM $CaCl_2$/1 mM $MgAc_2$ 0.05 M NaCl/10 mM β-mercaptoethanol buffer, pH 7.5 (buffer A) and then dialyzed against the same buffer overnight. The dialyzed sample was centrifuged at 40000 rev./min for 1 h and the supernatant was applied onto a DEAE cellulose column (4 cm × 40 cm) which had been equilibrated with buffer A. The column was first eluted with two bed vols. of buffer A and then with a linear salt gradient of 0.05—0.4 M NaCl. The total volume of the gradient was 1 liter. Both forms of phosphodiesterase usually came out together in a broad peak. The enzyme fractions were pooled and dialyzed overnight against buffer B (20 mM Tris,

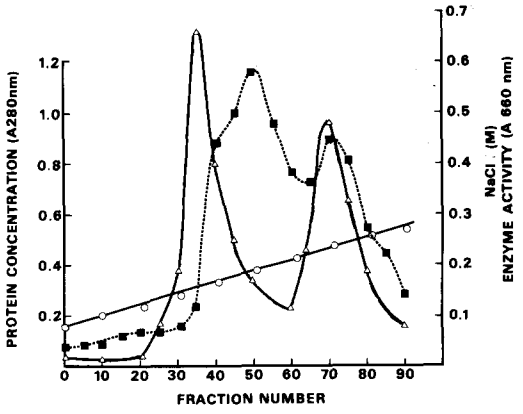

Fig. 1. Fractionation of bovine heart phosphodiesterase: an enzyme fraction from the first DEAE cellulose column (see Materials and Methods) was rechromatographed on DEAE cellulose column in buffer B. The column was eluted with an NaCl gradient of 0.05—0.35 M (○). Fractions were analyzed for protein concentration (■) and cyclic AMP phosphodiesterase activity (△) in the presence of saturating concentrations of Ca^{2+} and the protein activator.

1 mM imidazole, 0.1 mM EGTA, 1 mM $MgAc_2$ and 10 mM β-mercaptoethanol) and then applied to a DEAE cellulose column equilibrated with the same buffer. The column was eluted under the same conditions as the previous DEAE cellulose column, except that buffer B instead of buffer A was used. Phosphodiesterase was separated into two activity peaks (Fig. 1). They were pooled separately and stored in small aliquots at —20°C. The only difference between buffers A and B was that buffer A contained 10 μM $CaCl_2$ whereas buffer B contained 0.1 mM EGTA. The recovery of the peak I enzyme activity was usually around 5% of the total activity in the crude extract. The recovery of the peak II enzyme was more variable, ranging from 1 to 5% of the total activity found in the crude extract.

Assay of phosphodiesterase. Cyclic nucleotide phosphodiesterase was assayed at pH 7.5 and 30°C by one of the two methods. The first method which was the procedure of Butcher and Sutherland [22] was used mainly for the monitoring of the enzyme purification and other experiments where the substrate concentrations were in milimolar range. The method involved the conversion of the reaction product 5′-AMP or 5′-GMP to their respective nucleoside and inorganic phosphate by 5′-nucleotidase and followed by the colorimetric determination of the phosphate. Unless specified otherwise the reaction mixture (0.9 ml) contained 25 mM Tris, 25 mM imidazole, 3 mM $MgAc_2$, 1.2 mM cyclic nucleotide and 0.2 unit 5′-nucleotidase. Other components such as the enzyme, the activator and Ca^{2+} or EGTA were varied as indicated. The second assay method was based on the procedure of Thompson and Appleman [1] using cyclic [^{3}H]-nucleotides. The reaction conditions were the same as those of the first method except that 0.6 ml mixture was used and that the concentration of the nucleotide substrates was usually much lower. The reaction was terminated by boiling for 2 min. Then 1 ml of the ion exchange resin AG1-X2 slurry was added. The slurry was made by mixing one part of the resin with one part of 10 mM cold nucleoside (adenosine or guanosine). The reaction mixture was then centrifuged and an aliquot of the supernatant was counted in the scintillation

fluid. The fluid was prepared by dissolving 125 g naphthalene, 7.5 g of 2,5-diphenyloxazole and 0.375 g of 1,4-*bis*-2(-5 phenyloxazolyl)-benzene in 1 l of dioxane. Blank reactions were run concurrently with the test reactions for substrate blank correction. Another control using excess enzyme to hydrolyze the substrate completely was also carried out to correct for possible non-specific absorption of nucleosides by the anion exchange resin. The completion of the hydrolysis was always checked by thin-layer chromatography.

Results

Fig. 1 shows that bovine heart cyclic nucleotide phosphodiesterase is separated on DEAE cellulose column into two main activity peaks. These two peaks are labelled as P_I and P_{II} phosphodiesterases in the order of emergence from the column. The P_I enzyme is the protein activator activatable phosphodiesterase described previously [17], whereas the P_{II} enzyme is insensitive to the protein activator or to EGTA, the Ca^{2+} chelator. Table I shows that in the presence of sufficient amount of free Ca^{2+} in reaction mixtures, P_I enzyme is activated 9-fold by the protein activator. This enzyme activation is not observed if EGTA is present to chelate the free Ca^{2+} ions. In contrast to P_I phosphodiesterase, the P_{II} enzyme is not activated by the addition of protein activator. This lack of activation is not because the enzyme fraction is contaminated with protein activator, since EGTA has little effect on the activity of this enzyme. This difference in their responses to EGTA therefore suggests that the two activity peaks indeed represent two forms of the enzyme rather than the result of artifacts of the column elution. When the two enzyme fractions were pooled and rechromatographed separately on DEAE cellulose column, they each gave a single activity peak at the expected elution positions, thus further supporting the notion that they represent distinct molecular forms of the enzyme.

The relative amount of the two forms of cyclic nucleotide phosphodiesterase vary from preparations to preparations. The reason for this variation is not clear. For good and reproducible separations of these two forms on DEAE cellulose column, it is desirable to include 0.1 mM EGTA in the column elution buffer. In the absence of this chelating agent, P_I enzyme is often eluted at higher concentrations of NaCl. This is because Ca^{2+} promotes the interaction between the P_I enzyme and the highly negatively charged protein activator. This depend-

TABLE I

EFFECT OF EGTA AND THE PROTEIN ACTIVATOR ON PHOSPHODIESTERASE ACTIVITY

Enzyme form	Enzyme activity (units/mg) [a]		
	No addition	+ Protein activator [b]	+ Protein activator and EGTA [b,c]
P_I	0.30	2.8	0.31
P_{II}	0.43	0.48	0.45

[a] Enzyme activities were assayed with 1.2 mM cyclic AMP in the presence of 10 μM Ca^{2+}.
[b] Approximately 25 units of the purified protein activator were added.
[c] EGTA concentration in the reactions was 0.5 mM.

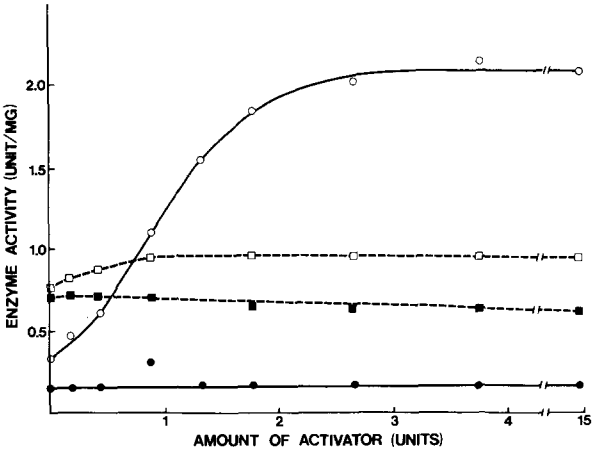

Fig. 2. Effect of protein activator on P_I phosphodiesterase: P_I phosphodiesterase was assayed in the presence of either 10 μM Ca^{2+} (○,□) or 0.1 mM EGTA (●,■) using either 1.2 mM cyclic AMP (○,●) or 1.2 mM cyclic GMP (□,■) as the substrate.

ence of the enzyme elution upon Ca^{2+} and EGTA has been used recently for the purification of P_I phosphodiesterase [18].

Both cyclic AMP and cyclic GMP may be hydrolyzed by either forms of bovine heart cyclic nucleotide phosphodiesterase. The P_I enzyme may be activated by Ca^{2+} and the protein activator when either cyclic nucleotide is used as the substrate (Fig. 2 and Table II). However, when cyclic GMP concentration is is in the millimolar range, the enzyme activation is very small. Pronounced activation of the P_I enzyme by the protein activator toward cyclic GMP hydrolysis was seen in low concentrations of the substrate (Table II). In contrast, the hydrolysis of cyclic AMP by this enzyme is strongly activated by the protein activator at all substrate concentrations tested. This differential activation by the protein activator on the hydrolyses of cyclic AMP and cyclic GMP arises from the difference in kinetic mechanisms of the enzyme activation. While the activated P_I phosphodiesterase is more active toward cyclic AMP than cyclic GMP, the non-activated enzyme shows a higher activity toward cyclic GMP (Fig. 2).

TABLE II

EFFECT OF SUBSTRATES ON THE ACTIVATION OF P_I PHOSPHODIESTERASE

Nucleotide substrate	Concentration (μM)	Enzyme activation [a] (% of basal activity)
Cyclic AMP	1200	590
	100	1020
	20	1340
Cyclic GMP	1000	122
	200	390
	20	585
	1	710

[a] Enzyme activations were carried out with 10 μM Ca^{2+} and 50 units of the protein activator.

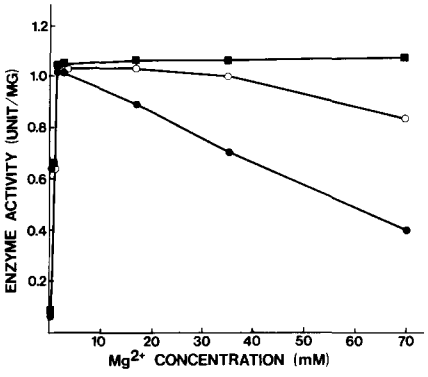

Fig. 3. Inhibition of P_I phosphodiesterase by Mg^{2+}: the enzyme was assayed in the presence of a saturating amount of the protein activator, 1.2 mM cyclic AMP and 10 (•), 30 (○) or 100 μM of Ca^{2+} (▪).

High concentrations of Mg^{2+} have been shown to inhibit mammalian cyclic nucleotide phosphodiesterases [24]. Fig. 3 shows that the activated P_I enzyme may be inhibited by high concentrations of Mg^{2+}. This inhibition is overcome by an increase in concentration of Ca^{2+}. These results suggest that the inhibition of cyclic nucleotide phosphodiesterase by Mg^{2+} results from the competition of this metal ion with Ca^{2+}. In contrast to the activated P_I phosphodiesterase, the non-activated P_I enzyme is not inhibited by high concentrations of Mg^{2+}, nor is P_{II} phosphodiesterase inhibited by Mg^{2+}.

Butcher and Sutherland [22] have shown that bovine heart cyclic nucleotide phosphodiesterase may be activated by imidazole. Fig. 4 shows that although all forms of the enzyme may be activated by this reagent, the imidazole activation is most pronounced with the activated P_I enzyme. The concentration of imidazole providing 50% stimulation to the activated P_I enzyme is around 7 mM. To study the mechanism of imidazole stimulation of the enzyme further, the dependence of P_I enzyme on the concentration of protein activator has been examined both in the absence and presence of 40 mM imidazole. Fig. 5

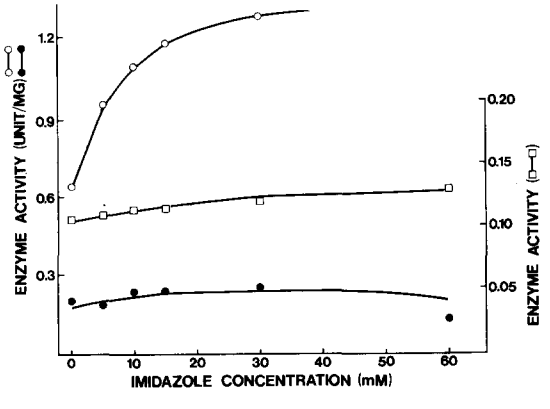

Fig. 4. Activation of phosphodiesterase by imidazole: P_I phosphodiesterase in the presence (○) and absence (•) of the protein activator and P_{II} phosphodiesterase (□) were assayed using cyclic AMP (1.2 mM) as the substrate.

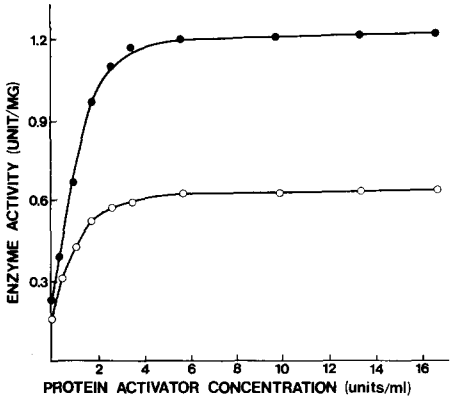

Fig. 5. Effect of imidazole on protein activator activation of phosphodiesterase: P_I phosphodiesterase was activated by varying amounts of protein activator in the absence (○) or presence (●) of 40 mM imidazole. Cyclic AMP (1.2 mM) was used as the substrate.

shows that the concentration of protein activator required to provide 50% of the maximal activation is not altered by imidazole, thus suggesting that the affinity of the protein activator towards the enzyme is not affected. This reagent however, enhances the maximal activation of the enzyme by the protein activator.

Ammonium ions have also been shown to activate cardiac cyclic nucleotide phosphodiesterase [25]. In many respects, activation of bovine heart cyclic nucleotide phosphodiesterase by NH_4^+ has been found to be similar to the imidazole activation. The major effect of the ions is to enhance the protein activator activation of the P_I phosphodiesterase by raising the maximal level of this activation by about 85%. The concentration of $(NH_4)_2SO_4$ that gave rise to 50% of the maximal stimulation was found to be 35 mM.

Kinetic properties of the two forms of bovine heart cyclic nucleotide phosphodiesterase have been examined using either cyclic AMP or cyclic GMP as the substrate. These results are summarized in Table III. The double reciprocal

TABLE III

KINETIC CONSTANTS OF BOVINE HEART PHOSPHODIESTERASES

Enzyme forms	K_m (mM) [c]		V (%) [a]	
	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
Non-activated				
P_I	1.2 —1.7	0.21 —0.33	20	30
Activated [b]				
P_I	0.16 —0.27	0.007—0.011	100	30
P_{II}	0.053—0.061	0.030—0.048	100	85

[a] Values are given as per cent of V values of P_{II} or activated P_I phosphodiesterase. Comparisons between V values of P_I and P_{II} phosphodiesterases are not meaningful.

[b] Reactions contained 20 μM Ca^{2+} and 20 units of the protein activator.

[c] Since the double reciprocal plot is non-linear, the K_m value was estimated as the substrate concentration at 50% V.

plots with respect to either cyclic AMP or cyclic GMP for P_I phosphodiesterase, either in the presence or in the absence of saturating amounts of the protein activator and Ca^{2+}, are linear, indicating the lack of cooperativity in the binding of the substrate to the enzyme. A comparison of the K_m and V values for the activated and the non-activated P_I phosphodiesterase with respect to cyclic AMP indicates that the enzyme activation by Ca^{2+} and the protein activator results from changes in both kinetic parameters. The V is increased about 5-fold by the activation and K_m is decreased by around 90%. When cyclic GMP instead of cyclic AMP is used as the substrate for P_I cyclic nucleotide phosphodiesterase, the enzyme activation by the protein activator and Ca^{2+} appears to follow a different kinetic mechanism. The V values for cyclic GMP hydrolysis by the activated and the non-activated P_I phosphodiesterase are approximately the same, whereas the K_m value with respect to cyclic GMP for the non-activated P_I phosphodiesterase is about 50 times higher than that of the activated enzyme. Thus, activation of the enzyme toward cyclic GMP by the protein activator and Ca^{2+} results solely from an enhancement of the apparent affinity of the enzyme toward this substrate.

A comparison between K_m values of P_I phosphodiesterase with respect to cyclic AMP and cyclic GMP, indicates that the enzyme has a much higher affinity for cyclic GMP than cyclic AMP either in the activated or the non-activated state. A similar observation has been made with the Ca^{2+}-activated phosphodiesterase of rat brain [28].

As has been pointed out in preceding paragraphs, P_{II} cyclic nucleotide phosphodiesterase is not activated by the protein activator and Ca^{2+}. This lack of response of the P_{II} enzyme to the activators has been tested under many different conditions; using either cyclic AMP or cyclic GMP as the substrate at concentrations ranging from μM to 4 mM. In addition to their differences in regulatory properties, the two forms of cyclic nucleotide phosphodiesterase also exhibit different kinetic properties and relative substrate specificity. Double reciprocal plots for P_{II} phosphodiesterase with respect to either cyclic AMP (Fig. 6) or cyclic GMP (Fig. 7) are shown. When cyclic GMP is used as the sub-

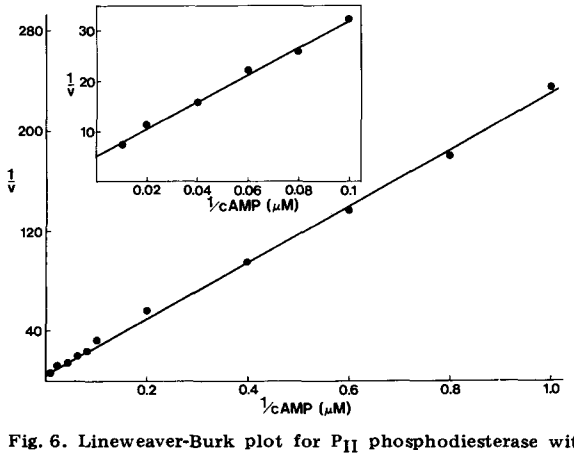

Fig. 6. Lineweaver-Burk plot for P_{II} phosphodiesterase with respect to cyclic AMP. Inset: data at high substrate concentrations were replotted on an expanded scale.

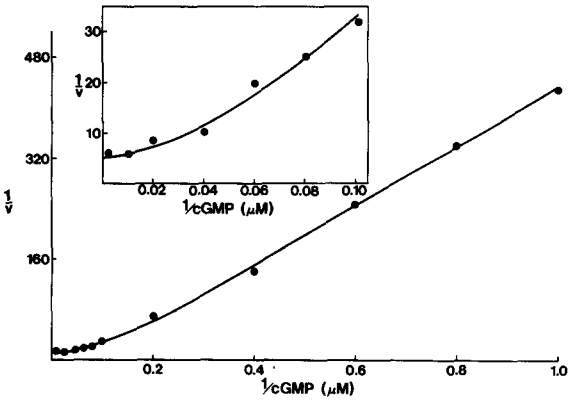

Fig. 7. Lineweaver-Burk plot for P_{II} phosphodiesterase with respect to cyclic GMP. Inset: data at high substrate concentrations plotted at an expanded scale.

strate, the plot shows a pronounced upward curvature, suggesting the presence of homotropic cooperativity in the enzyme with respect to this substrate. There are no significant homotropic interactions in the enzyme when cyclic AMP is the substrate. While P_I phosphodiesterase shows a much higher affinity for cyclic GMP than cyclic AMP, the P_{II} enzyme has no pronounced preference in the binding of the two substrates. The K_m values of the P_{II} enzyme for cyclic AMP and cyclic GMP are in the same order of magnitude, although that for cyclic AMP is always somewhat higher (Table III). Table III also shows that the V values of P_{II} phosphodiesterase for the two substrates are similar.

For each of the kinetic constants presented in Table IV, three determinations were carried out, usually with different enzyme preparations. The K_m values obtained with different experiments were in good agreement. The range of their values were reported in Table IV. In different enzyme preparations, the absolute V values were found to vary by as much as 200%. This is because the enzyme used was not pure and the degree of purification varied from preparation to preparation. However, the relative V values for any one preparation of the enzyme are constant. The values for V in Table IV may be considered as representative data.

TABLE IV

KINETICS OF INHIBITION OF PHOSPHODIESTERASES

Enzyme form	Inhibitor	Type of inhibition [a]	Inhibition constant [a] (mM)
Non-activated			
P_I	Cyclic AMP	competitive	1.4
	Cyclic GMP	competitive	0.29
Activated			
P_I	Cyclic AMP	competitive	0.33
P_{II}	Cyclic AMP	noncompetitive	0.075
	Cyclic GMP	noncompetitive	0.2

[a] The kinetic analyses were carried out by Dixon plots.

In a further characterization of the two forms of bovine heart cyclic nucleotide phosphodiesterase, inhibition kinetics for the enzyme were analyzed by Dixon plots. The results of these studies are summarized in Table IV. Both the activated and non-activated phosphodiesterase are competitively inhibited by cyclic AMP when cyclic GMP is used as the substrate. The K_i values of cyclic AMP are seen to be comparable to the K_m values of cyclic AMP of the respective enzyme forms (Table III). Similarly, cyclic GMP competitively inhibits the non-activated P_I phosphodiesterase when cyclic AMP is used as the substrate. The inhibition constant also agrees reasonably well with the K_m of the non-activated P_I phosphodiesterase with respect to cyclic GMP. The Dixon plot of cyclic AMP inhibition of the activated P_I phosphodiesterase-catalyzed cyclic GMP hydrolysis also suggested competitive inhibition with a K_i similar to the K_m of cyclic AMP (Table IV vs. Table III). The observation that K_i values of the cyclic nucleotide are similar to the respective K_m values of the enzyme, supports the view that the two cyclic nucleotides are hydrolyzed at the same enzyme site.

In contrast to the P_I enzyme, P_{II} phosphodiesterase is inhibited by cyclic AMP or cyclic GMP, non-competitively with respect to the alternate nucleotide as the substrate. The K_i values of the P_{II} enzyme for cyclic AMP and for cyclic GMP may be obtained from the Dixon plots as 0.075 mM and 0.2 mM respectively. These values, and especially that for cyclic GMP, are significantly different from the K_m values of the respective cyclic nucleotide substrates (Table III vs. Table IV). Thus, it seems that the kinetic properties of the P_{II} enzyme are much more complex than those of the P_I enzyme. The results are not incompatible with the presence of separate enzymes in this enzyme fraction or the existence of separate active sites for these two nucleotides on the same enzyme. Further studies are certainly needed for this form of the enzyme.

Discussion

The existence and fractionation of multiple forms of bovine heart cyclic nucleotide phosphodiesterase have been reported previously from several laboratories [13—17]. However, in view of the recent observation that the bovine heart enzyme may be activated by the protein activator and Ca^{2+} [21], it appears that a further characterization of multiple phosphodiesterases in this tissue is desirable.

The present work demonstrates the separation of bovine heart cyclic nucleotide phosphodiesterase into two fractions. Since the two enzyme fractions exhibit very different kinetic and regulatory properties, it is suggested that they represent different forms of the enzyme, designated as P_I and P_{II} phosphodiesterases. Only the P_I enzyme depends on Ca^{2+} and the protein activator for full activity. However, the partially purified P_I enzyme always exhibits significant enzyme activity in the absence of the added protein activator. This "basal activity" is not due to contamination of the protein activator in this enzyme fraction, since it is not significantly changed by EGTA. In addition, unlike the activity of P_I phosphodiesterase, this basal activity is not stimulated by imidazole nor inhibited by high concentrations of Mg^{2+}. Although many of these properties, e.g. the lack of responses to EGTA, imidazole and high con-

centrations of Mg^{2+}, are similar to those exhibited by P_{II} phosphodiesterase, the basal activity of the P_I enzyme cannot be attributed to a contamination of P_{II} phosphodiesterase. This is because the P_{II} enzyme and this basal activity exhibit very different kinetic properties.

Both the cyclic AMP and the cyclic GMP hydrolysis catalyzed by P_I phosphodiesterase can be activated by the protein activator. However, the kinetic mechanisms for these activations are different. The cyclic GMP hydrolysis is activated by the protein activator according to a "K" system; e.g. only the substrate affinity of the enzyme is affected by the activators, whereas the activation of cyclic AMP hydrolysis involves changes in both K_m and V. Although the reason for this difference in the activation mechanism is not known, the observation suggests that these nucleotide substrates may play a role in defining the final conformation of the activated enzyme. For other enzyme systems, it has been observed that kinetic mechanism of allosteric modulations may be dependent of the structure of ligands [26,27].

While P_{II} cyclic nucleotide phosphodiesterase possesses similar K_m values for cyclic AMP and cyclic GMP, the P_I enzyme shows a preferential binding for cyclic GMP over cyclic AMP. The result suggests that the P_I enzyme may be responsible for cyclic GMP hydrolysis in vivo. Similar suggestions have been made by Kakiuchi et al. [28] and Brostrom et al. [29] for the activator dependent phosphodiesterase in mammalian brains. The latter investigators [29] have found that the same protein activator may also activate brain adenyl cyclase and suggested that this activator regulates intracellular concentrations of both nucleotides in a concerted manner.

As has been reviewed by Appleman et al. [30], kinetically distinguishable cyclic nucleotide phosphodiesterase have been found in many animal tissues. Usually, a tissue contains both high K_m (about 100 μM) and low K_m (around 1 μM) cyclic AMP hydrolyzing activity and a cyclic GMP diesterase activity with a K_m in the range of 10—100 μM. The result shown in Table I suggests that neither P_I nor P_{II} phosphodiesterase represent low K_m cyclic AMP phosphodiesterase. This is not suprising since the low K_m enzyme is often found in association with the membrane fraction [30]. The lack of low K_m cyclic AMP phosphodiesterase in the soluble extract of bovine heart has also been shown by other investigators [14,15].

Goren and Rosen [14] have fractionated bovine heart phosphodiesterase on a Prep-Disc electrophoretic column into two enzyme fractions: phosphodiesterases I and II. The two forms of the enzyme exhibit similar kinetic properties. They possess K_m values of about $3 \cdot 10^{-4}$ and $5 \cdot 10^{-5}$ M for cyclic AMP and cyclic GMP respectively. In comparison with the kinetic properties of the P_I and P_{II} phosphodiesterases (Table I), the two forms of the enzyme appear similar to the activated P_I phosphodiesterase in terms of K_m values for cyclic AMP, but to P_{II} enzyme in terms of K_m of cyclic GMP. However, since they have also shown that their purified enzyme may be activated by the protein activator, it is more likely that the enzyme forms are identical to P_I.

Hrapchak and Rasmussen [15] have fractionated bovine heart cyclic nucleotide phosphodiesterase on DEAE cellulose column into two fractions: phosphodiesterase I and II. Although phosphodiesterases I and II may be identified with P_I and P_{II} phosphodiesterases on the basis of their elution from the DEAE

cellulose column, their kinetic properties are very similar, unlike those of P_I and P_{II} enzymes. The K_m values for cyclic AMP of phosphodiesterases I and II are respectively 30 and 60 μM. Thus, both are similar to P_{II} phosphodiesterase. The K_m values for cyclic GMP were not reported for these two forms of phosphodiesterase.

From the above discussions, it seems that an unequivocal identification of the multiple forms of bovine heart phosphodiesterase reported from different laboratories are difficult. Undoubtedly, part of the difficulty is due to the different conditions used by the different investigators in the characterization of the multiple enzyme forms. Also, the methods used for enzyme preparation may cause alterations in kinetic properties of the enzyme and therefore contribute to the difficulty in enzyme identifications. During this study, we have observed that the P_I enzyme may become less responsive to the protein activator upon purification.

Some of the best kinetic characterizations of multiple forms of cyclic nucleotide phosphodiesterase have been from Appleman's laboratory [11]. They have fractionated the rat liver enzyme on DEAE cellulose column into three fractions: DI, DII and DIII phosphodiesterase. Of these DIII is a membrane-bound, low K_m, cyclic AMP phosphodiesterase. Interestingly, the two soluble forms of the enzyme, DI and DII phosphodiesterases are similar in many respects to P_I and P_{II} phosphodiesterases, respectively, of bovine heart. The only major difference between the P_I and DI phosphodiesterases is that only the P_I phosphodiesterase can hydrolyze cyclic AMP. The similarity between the bovine heart and rat liver enzymes suggests that similar forms of the enzyme are to be expected in different tissues.

Upon completion of this work, a report by Appleman and Terasaki [31] appeared showing that rat heart also contained DI phosphodiesterase. In addition, the DI enzyme of rat heart depended on Ca^{2+} and the protein activator for full activity.

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