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PROPERTIES OF THE ACTIVATOR-DEPENDENT CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM BOVINE HEART

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Summary

In the absence of activator, the activator-dependent cyclic nucleotide phosphodiesterase (EC 3.1.4.-) from bovine heart hydrolyzed 3.2-fold more cyclic GMP than cyclic AMP when assayed with 10^{-6} M substrate in the presence of 5 mM Mg^{2+} and 10 μM Ca^{2+}. The addition of saturating amounts of phosphodiesterase activator increased the hydrolysis of cyclic GMP 8-fold and cyclic AMP 6-fold. The pH maxima of the enzyme was rather broad from 6.0 to 7.0 for the hydrolysis of both cyclic GMP and cyclic AMP in the absence or presence of phosphodiesterase activator. Substrate specificity of the enzyme in the absence or presence of phosphodiesterase activator varied depending on the divalent metal used to support activity in the absence of activator. The enzyme preferentially hydrolyzed cyclic GMP in the presence of Mg^{2+} while little substrate specificity was observed in the presence of Mn^{2+}, Zn^{2+}, Co^{2+} or Ni^{2+}. The magnitude of the increase in enzyme activity due to activator and Ca^{2+} varied depending on the divalent metal used to support enzyme activity without activator. The addition of activator increased the V of cyclic AMP hydrolysis 1.7-fold while causing no significant change in the K_m for cyclic AMP. Two apparent K_m values for cyclic GMP (1 and 15 μM) were noted in the absence of activator and upon the addition of activator a single apparent K_m (3 μM) was observed with a V approx. 2-fold above that in the absence of activator. Cyclic AMP competitively inhibited the hydrolysis of cyclic GMP with a K_i of 50 μM while cyclic GMP non-competitively inhibited the hydrolysis of cyclic AMP with a K_i of 1.8 μM. The results suggest there may be two or more binding sites for cyclic GMP on the enzyme and possibly only one binding site for cyclic AMP.

Introduction

Multiple molecular forms of cyclic nucleotide phosphodiesterase (EC 3.1.4.-) varying in several catalytic properties have been noted in many tissues [1—14].

Cheung [15,16] initially demonstrated a brain phosphodiesterase which required a heat-stable protein, phosphodiesterase activator, for maximal activity. In addition to brain [15—28], other tissues including heart [29—33], coronary arteries [34] and liver [23] have been shown to have an activator-dependent phosphodiesterase. Teo and Wang [33] have shown that a purified sample of the activator protein from bovine heart binds Ca^{2+}, suggesting that a complex of Ca^{2+} and the protein is the true activator of phosphodiesterase. Teshima and Kakiuchi [35] demonstrated the formation of an active enzyme-activating factor complex in the presence of Ca^{2+}; in the absence of Ca^{2+}, it dissociated into an inactive form of the enzyme and activating factor. Most early studies of the activator-dependent enzymes examined their activity with 10^{-4} M cyclic AMP or cyclic GMP as substrate [15—17,19—22,26,29,30,33] and under these conditions the enzymes were termed cyclic AMP-phosphodiesterases. Recent studies of the enzymes from brain [18,23—25,27,28,36], liver [23], coronary arteries [34] and aorta [37] with 10^{-6} M cyclic AMP or cyclic GMP as substrate have indicated that under these conditions the enzymes are more specific for cyclic GMP. We recently reported on the presence of a rather cyclic GMP-specific phosphodiesterase in bovine heart [38] and in this report using a more purified enzyme and low substrate levels we describe some properties of the enzyme.

Materials and Methods

Cyclic AMP, cyclic GMP and *Crotalus atrox* venom were purchased from Sigma. Cyclic [^{3}H]AMP and cyclic [^{3}H]GMP were purchased from Amersham/Searle.

Phosphodiesterase activity was assayed by a procedure adapted from Russell et al. [12]. An appropriate dilution of enzyme was incubated in 40 mM Tris · HCl, pH 7.4, 5 mM $MgCl_2$ containing $1 \cdot 10^{-8}$—$3 \cdot 10^{-8}$ M cyclic [^{3}H]AMP or cyclic [^{3}H]GMP in a total volume of 1 ml. When higher concentrations of cyclic nucleotides (usually 10^{-6} M cyclic AMP or cyclic GMP) were required, the indicated amounts of unlabelled cyclic nucleotides were included. In several experiments, 10 μM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) was added to remove trace metals (Fe^{2+} and Cu^{2+}) which inhibit enzyme activity and also to remove approx. 0.5 μM Ca^{2+} present in the enzyme, activator and reagents. Other additions to or alterations of this assay procedure are as indicated in the legends to the figures and the table. After 10 min at 30°C, the reaction was terminated by boiling for 3 min and the sample processed as described [12]. The amount of phosphodiesterase used was adjusted so that no more than 15% of the cyclic nucleotide was hydrolyzed during the incubation time.

Cyclic nucleotide phosphodiesterase activity from bovine heart was initially resolved into three peaks by a procedure involving ammonium sulfate precipitation and DEAE-cellulose chromatography as described previously [38]. Peak I activity, which represented approximately one-third of the total activity for cyclic AMP and cyclic GMP hydrolysis at this step, was stimulated by phosphodiesterase activator in the standard incubation mixture supplemented with Ca^{2+} [38]. Peak I enzyme from 275 g of bovine heart was dialyzed against

15 l of 10 mM potassium phosphate buffer, pH 7.4, and 2 mM 2-mercaptoethanol. The dialysate was rechromatographed by applying it to a column of DEAE-cellulose (4 × 24 cm) which had been equilibrated with the homogenization buffer. The loaded column was eluted with a 1.0 l gradient of 20—400 mM potassium phosphate buffer, pH 7.4, and 4 mM 2-meracptoethanol. Approx. 10-ml fractions were collected and 10-μl aliquots were removed for assay with 10^{-6} M cyclic AMP or cyclic GMP as substrate in the standard procedure. The active peak was pooled as indicated in Fig. 1 and concentrated using a PM-10 membrane in an Amicon ultrafiltration chamber. As indicated under Results, this enzyme preparation was found to be free of phosphodiesterase activator.

Phosphodiesterase activator was prepared from bovine liver as described previously [38].

Results

The pattern of elution of the activator-dependent cyclic nucleotide phosphodiesterase of bovine heart following rechromatography on a DEAE-cellulose column is presented in Fig. 1. The activity for both cyclic AMP and cyclic GMP hydrolysis co-chromatographed, with the enzyme hydrolyzing 2—3 times more cyclic GMP than cyclic AMP under the standard assay conditions.

Linearity of 10^{-6} M cyclic GMP or cyclic AMP hydrolysis was examined as a function of both time and enzyme concentration in the absence and presence of phosphodiesterase activator.It was noted that the reaction was linear up to 30 min under all conditions, provided that the amount of substrate hydrolyzed did not exceed 20%. The lack of linearity above this level of hydrolysis was

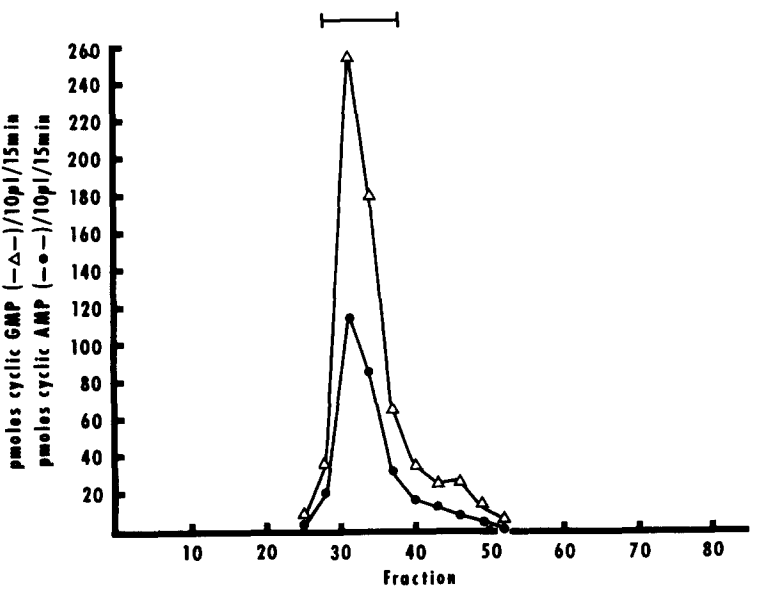

Fig. 1. DEAE-cellulose re-chromatography of bovine heart peak 1 phosphodiesterase. Details of the chromatographic procedure are given under Materials and Methods. Aliquots (10 μl) of the indicated fractions were assayed with 10^{-6} M cyclic GMP or cyclic AMP by the standard procedure in the absence of added phosphodiesterase activator. The peak tubes were pooled as indicated.

apparently due to substrate depletion and not end-product inhibition as the addition of 5'-AMP or 5'-GMP (10^{-8}—10^{-4} M) did not alter the hydrolysis of cyclic AMP or cyclic GMP, respectively.

The pH maxima for the hydrolysis of both cyclic AMP and cyclic GMP in the presence of phosphodiesterase activator was approximately pH 6.4, however, the activity did not vary greatly from pH 5.9 to 7.4. A similar pH profile of cyclic GMP hydrolysis was observed in the absence of activator while that for cyclic AMP was quite flat from pH 5.9 to 8.2.

The addition of increasing amounts of phosphodiesterase activator resulted in the hydrolysis of increasing amounts of both cyclic GMP and cyclic AMP when assayed with 10^{-6} M substrate (Fig. 2). When fully activated the enzyme hydrolyzed 8-fold more cyclic GMP and 6.5-fold more cyclic AMP than in the absence of activator. As previously shown [33,35], the activity of phosphodiesterase activator required the addition of calcium. The calcium requirement for half-maximal activator activity was approximately the same for cyclic AMP and cyclic GMP hydrolysis (Fig. 3). Kakiuchi et al. [18] noted with the enzyme from rat cerebral cortex that the rate of cyclic GMP hydrolysis was stimulated by a 50% lower concentration of Ca^{2+} than that of cyclic AMP hydrolysis. The activity of added phosphodiesterase activator with the bovine heart enzyme could be eliminated by the addition of EGTA which rather selectively chelates calcium. The addition of 10—150 μM EGTA, pH 7.4, in the absence of added activator did not decrease the hydrolysis of cyclic AMP or cyclic GMP demonstrating that phosphodiesterase activator was not a contaminant of the enzyme preparation.

The metal requirements of the hydrolysis of 10^{-6} M cyclic AMP and cyclic GMP were examined in the absence and presence of phosphodiesterase activator (Table I). As Ca^{2+} is required for activator activity and Ca^{2+} alone does not support enzymatic activity, 20 μM $CaCl_2$ and 10 μM EGTA, pH 7.4, were added to all samples both in the absence and presence of activator. The con-

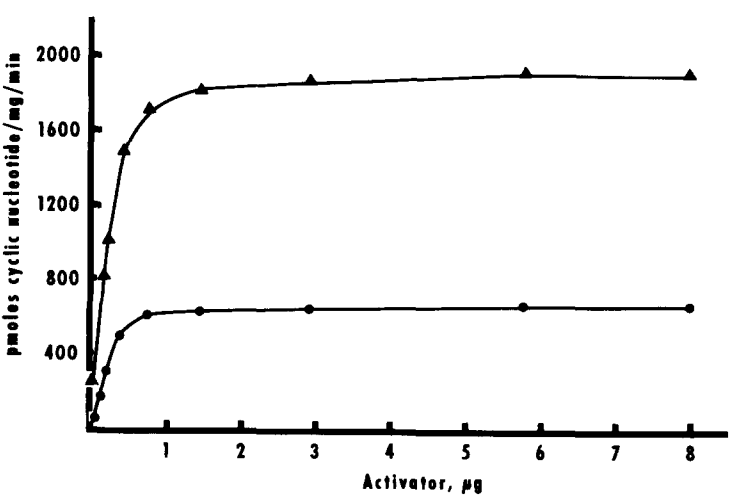

Fig. 2. Effect of increasing concentrations of phosphodiesterase activator on the hydrolysis of 10^{-6} M cyclic GMP (▲) and cyclic AMP (●). All samples contained 10 μM EGTA, pH 7.4, and 20 μM $CaCl_2$.

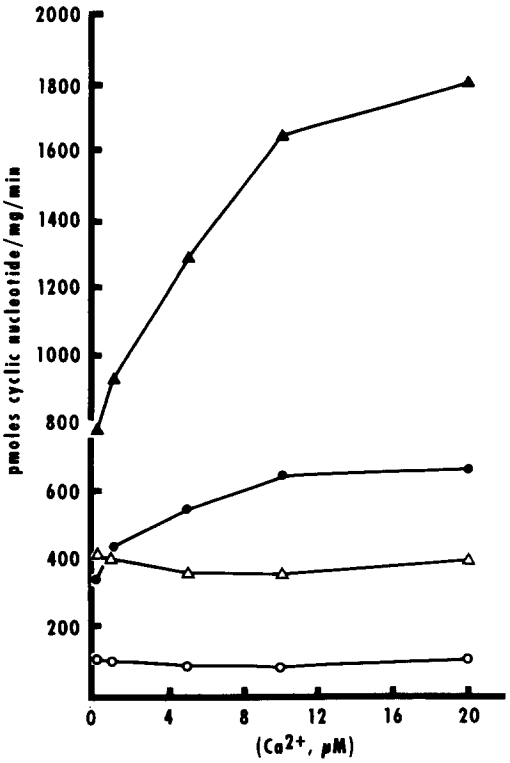

Fig. 3. Effect of increasing concentrations of $CaCl_2$ on the hydrolysis of 10^{-6} M cyclic GMP (▲) and cyclic AMP (●) in the presence of phosphodiesterase activator (5 μg). Open symbols indicate hydrolysis in the absence of phosphodiesterase activator.

centration of metals used were those giving maximal enzyme activity in the absence of activator. Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} supported enzyme activity in the absence of activator, however, the substrate specificity of the enzyme varied with the individual metals. For example, the enzyme hydrolyzed 3.2-fold more cyclic GMP than cyclic AMP in the presence of Mg^{2+} while the enzyme generally hydrolyzed more cyclic AMP and less cyclic GMP in the presence of other metals resulting in a rather non-specific enzyme. Cu^{2+} and Fe^{2+} did not support enzyme activity in the absence of activator. In the presence of phosphodiesterase activator, the divalent metals used to support unstimulated activity influenced both the substrate specificity of the enzyme and the degree of stimulation by activator. The enzyme hydrolyzed 4.3-fold more cyclic GMP than cyclic AMP with Mg^{2+} in the presence of phosphodiesterase activator. With Mn^{2+}, the enzyme was relatively non-specific as the level of cyclic GMP hydrolysis was 46% of that with Mg^{2+} while cyclic AMP hydrolysis increased 67%. The enzyme was also relatively non-specific with Zn^{2+} and Co^{2+} and somewhat cyclic GMP specific with Ni^{2+} in the presence of phosphodiesterase activator. The greatest stimulation of cyclic AMP and cyclic GMP hydrolysis by activator occurred with Mn^{2+} and Mg^{2+}, respectively, while the smallest stimulation of hydrolysis of both substrates occurred with Zn^{2+}.

TABLE I

EFFECTS OF DIVALENT METALS ON THE HYDROLYSIS OF 10^{-6} M CYLIC GMP OR CYCLIC AMP IN THE ABSENCE AND PRESENCE OF A SATURATTING AMOUNT OF PHOSPHODIESTERASE ACTIVATOR

All samples contained 10 μM EGTA, pH 7.4 and 20 μM $CaCl_2$. The results are the mean ± S.E. of 12 determinations from three experiments.

Metal	Minus activator			Plus activator			Plus/minus activator	
	Cyclic GMP	Cyclic AMP	Cyclic GMP / Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP / Cyclic AMP	Cyclic GMP	Cyclic AMP
Control	20 ± 2.0	15 ± 1.2	1.33	24 ± 2.1	22 ± 2.3	1.09	1.20	1.46
Mg^{2+}(5 mM)	310 ± 14.2	97 ± 3.6	3.20	2548 ± 85.6	592 ± 22.4	4.30	8.22	6.10
Mn^{2+}(1 mM)	144 ± 5.1	118 ± 2.9	1.22	1163 ± 35.8	990 ± 38.4	1.17	8.10	8.38
Zn^{2+}(100 μM)	166 ± 6.3	123 ± 6.1	1.35	558 ± 23.6	540 ± 37.8	1.03	3.36	4.39
Ni^{2+}(100 μM)	194 ± 5.8	148 ± 5.9	1.31	1274 ± 41.8	592 ± 28.4	2.15	6.56	4.00
Co^{2+}(100 μM)	238 ± 6.4	182 ± 8.1	1.30	1265 ± 64.3	1333 ± 71.6	0.95	5.35	7.32

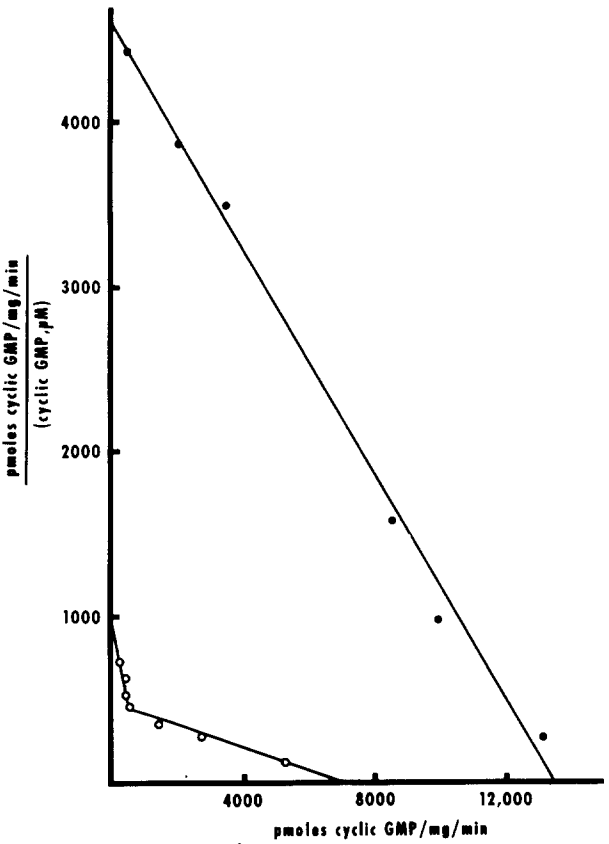

Fig. 4. Hofstee plot of cyclic GMP (10^{-7}—10^{-4} M) hydrolysis in the absence (○) and presence (●) of phosphodiesterase activator (5 μg). All samples contained 10 μM EGTA, pH 7.4, and 20 μM $CaCl_2$.

The apparent K_m of the enzyme for cyclic GMP and cyclic AMP was examined in the absence and presence of saturating amounts of phosphodiesterase activator. Hofstee plots [39] of enzyme activity against a wide range of cyclic GMP concentrations (10^{-7}—10^{-4} M) indicate two apparent K_m values (1 and 15 μM) for cyclic GMP in the absence of activator (Fig. 4). A linear plot was obtained in the presence of activator indicating a K_m of 3 μM for cyclic GMP and a V 2-fold above that in the absence of activator. Similar results with cyclic GMP as substrate have been observed with partially purified activator-dependent phosphodiesterases from bovine [27] and rat [18] cerebral cortex. Linear plots of cyclic AMP hydrolysis were obtained in the absence and presence of phosphodiesterase activator (Fig. 5). The activator increased the V 1.7-fold while causing no significant change in the apparent K_m (34 μM) for cyclic AMP. Teo et al. [32] noted in an earlier study with a less purified enzyme from bovine heart that the activator decreased the apparent K_m for cyclic AMP from 1.4 to 0.4 mM while increasing the V 3-fold. Goren and Rosen [30] reported that the activator decreased the apparent K_m of the bovine heart enzyme for cyclic AMP from $5.2 \cdot 10^{-4}$ to $2.2 \cdot 10^{-4}$ M and increased the V 75%. These earlier studies involved high substrate levels because of the use of a relatively insensi-

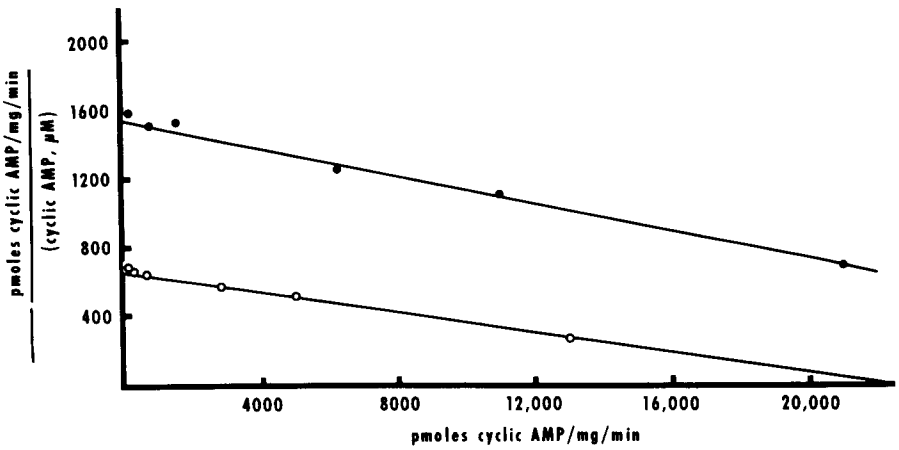

Fig. 5. Hofstee plot of cyclic AMP (10^{-7}—10^{-4} M) hydrolysis in the absence (○) and presence (●) of phosphodiesterase activator (5 μg). All samples contained 10 μM EGTA, pH 7.4, and 20 μM $CaCl_2$.

tive spectrophotometric assay and this apparently is the major reason for the differences from the present results.

It has been noted with the enzyme from rat cerebral cortex [18] that the substrate specificity of the enzyme differed at low and high substrate levels. The enzyme from bovine heart similarly hydrolyzed about 4-fold more cyclic GMP than cyclic AMP in the presence of phosphodiesterase activator with 10^{-6} M substrate but hydrolyzed 3-fold more cyclic AMP with $5 \cdot 10^{-5}$ M substrate. This change in substrate specificity at low and high substrate levels can be explained by the higher K_m and 3-fold higher V for cyclic AMP as compared with cyclic GMP. In the absence of activator the enzyme hydrolyzed 3.2-fold more cyclic GMP than cyclic AMP with 10^{-6} M substrate but hydrolyzed 1.5-fold more cyclic AMP than cyclic GMP with $5 \cdot 10^{-5}$ M substrate.

If the activator-dependent phosphodiesterase from bovine heart consists of one enzyme with a common catalytic site for both cyclic AMP and cyclic GMP, the two nucleotides should competitively inhibit the hydrolysis of each other with relative inhibitory potencies predictable from their apparent K_m values as substrates. Both nucleotides were indeed inhibitors of the hydrolysis of each other by the enzyme + activator with cGMP more potent in inhibiting cAMP hydrolysis than the converse. Dixon plots [40] of the data demonstrated, however, that cyclic AMP competitively inhibited the hydrolysis of cyclic GMP with a K_i of 50 μM while cyclic GMP non-competitively inhibited the hydrolysis of cyclic AMP with a K_i of 1.8 μM.

Discussion

Activator-dependent cyclic nucleotide phosphodiesterase from bovine heart is a relatively cyclic GMP-specific enzyme when assayed in the presence of optimal levels of Mg^{2+} and 10^{-6} M cyclic GMP or cyclic AMP. However, the enzyme was less cyclic GMP specific in the presence of other divalent metals. Tipton and Cooke [41] reported that the levels of divalent metals in the

human heart are 7.8 mM Mg^{2+}, 1.3 mM Ca^{2+}, 1 mM Fe^{2+}, 0.5 mM Zn^{2+}, 60 μM Cu^{2+}, 4.6 μM Mn^{2+}, 1 μM Ni^{2+} and 1 μM Co^{2+}. The physiological significance of the differences observed in the substrate specificity of the enzyme in the presence of the various metals is unknown since the concentration of these metals that would be free for interaction with this phosphodiesterase are unknown. As suggested by others, Ca^{2+} probably has an important role in regulating not only the activity of the activator-dependent cyclic nucleotide phosphodiesterase of various tissues [15—37], but also may regulate the activity of guanylate cyclase [42—44] and adenylate cyclase [45—49]. The high in vivo levels of Mg^{2+} and Zn^{2+} suggest that these divalent metals may be able to influence enzyme activity provided, of course, that a significant amount of the metal is free for interaction with the enzyme. The low in vivo levels of Ni^{2+}, Co^{2+} and Mn^{2+} suggest that these metals probably have little physiological role in this system unless they are compartmentalized in association with the enzyme. Schultz and Schultz [50] have recently noted that the addition of Mg^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} or Mn^{2+} (2 mM) to an isolated rat ductus deferens preparation pre-incubated without Ca^{2+} caused 50—1500% increases in the intracellular cyclic GMP levels. Part of the increase could be due to the metals substituting for Ca^{2+} in the regulation of guanylate cyclase activity [42—44] but the metals may also have altered the activity of one or more cyclic nucleotide phosphodiesterases.

The activator-dependent phosphodiesterase from bovine heart displays non-linear kinetics in the hydrolysis of cyclic GMP but not cyclic AMP when assayed with 5 mM Mg^{2+} and 10 μM $CaCl_2$ in the absence of phosphodiesterase activator. Non-linearity of cyclic GMP hydrolysis has also been observed with the enzymes from rat [18] and bovine [27] cerebral cortex when assyed under similar conditions. However this was not observed with the enzymes from rat brain [24], pig coronary arteries [34] or rat liver [12]. Linear kinetics of cylic GMP hydrolysis in the presence of phosphodiesterase activator have been observed in this and previous studies [18,24,27,34]. Several studies [24,34] have suggested that cyclic GMP and cyclic AMP have the same catalytic site, as both cyclic nucleotides competitively inhibited the hydrolysis of the other with K_i values rather close to their own apparent K_m values as substrates for the enzyme. However, in this study cyclic GMP non-competitively inhibited the hydrolysis of cyclic AMP in the presence of phosphodiesterase activator with a K_i about the same as its apparent K_m while cyclic AMP competitively inhibited the hydrolysis of cyclic GMP with a K_i about twice its apparent K_m. The results suggest that the activator-dependent phosphodiesterase from bovine heart may have more than one binding site for cyclic GMP while there may only be one binding site for cyclic AMP.

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