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PROTEIN ACTIVATOR OF CYCLIC AMP PHOSPHODIESTERASE AND CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN BOVINE RETINA AND BOVINE LENS

ACTIVITY, SUBCELLULAR DISTRIBUTION AND KINETIC PARAMETERS

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Summary

We have examined the activity of cyclic AMP phosphodiesterase, cyclic GMP phosphodiesterase and the protein activator of cyclic AMP phosphodiesterase in various anatomic and subcellular fractions of the bovine eye. Cyclic GMP hydrolysis was 1.6—12 times faster than hydrolysis of cyclic AMP in the subcellular fractions of the retina and in the precipitate of the rod outer segment. An opposite pattern was seen in the bovine lens, where the hydrolysis of cyclic AMP occurred 17 and 169 times faster than that of cyclic GMP in the supernatant and precipitate of lens, respectively.

The activity of cyclic AMP phosphodiesterase was not affected by ethyleneglycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid in any fractions except in the retinal supernatant, suggesting that the phosphodiesterase exists primarily as a Ca^{2+}-independent, activator-independent form. However, the protein activator of cyclic AMP phosphodiesterase existed in all fractions examined.

A complex kinetic pattern was observed for both cyclic AMP and cyclic GMP hydrolysis by the 105000 × *g* lens supernatant. The Michaelis constants for both cyclic AMP ($1.3 \cdot 10^{-6}$ and $9.2 \cdot 10^{-6}$ M) and cyclic GMP ($1.04 \cdot 10^{-6}$ and $1.22 \cdot 10^{-5}$ M) appeared to be similar.

Introduction

A specific protein activator of cyclic AMP phosphodiesterase, originally identified in bovine brain [1], has now been described in many other tissues [2—4] including the retina [5]. High activity of cyclase and phosphodiesterase has also been demonstrated in retina and in rod outer segment [6—11] indicat-

ing the probability that cyclic nucleotides play a special role in the visual process. It has been reported that cyclic nucleotides regulate the differentiation [12], and function of the retina [13—15].

The presence of a protein activator specific for cyclic AMP phosphodiesterase raises the possibility of physiological regulation of the enzyme by the protein activator in the eye tissues. Despite its potentially important role in regulating cyclic nucleotides levels, the protein activator from the retina has only recently been the subject of investigation [5]. The present study was undertaken to determine the activity and distribution of the protein activator and phosphodiesterase in bovine retina, rod outer segment and lens and to analyze the kinetic parameters of the soluble phosphodiesterase from bovine lens.

Materials and Methods

Chemicals and reagents. Bovine serum albumin, snake venom (*Crotalus atrox*), ethyleneglycol bis(β-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA), cyclic AMP, and cyclic GMP were purchased from Sigma. Cellulose sheet was obtained from Eastman. Tritiated cyclic AMP and cyclic GMP (spec. act. 36.6 Ci/mmol and 8.28 Ci/mmol, New England Nuclear) was purified by thin layer chromatography on cellulose sheet with isopropanol/NH_4OH/H_2O (7 : 1 : 2, v/v). The resin IRP-58 (200—400 mesh, Rohm and Haas) was washed with 0.5 N NaOH, glass-distilled water, 0.5 M HCl and then thoroughly with glass-distilled water until the mixture was at pH 4. It was used as a slurry of one part resin to two parts water (v/v).

Preparation of bovine brain phosphodiesterase. Activator-deficient bovine brain phosphodiesterase was prepared as previously described [1]. The enzyme was purified by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography. This enzyme had 10% basal activity dependent on the presence of Ca^{2+} and had a specific activity of 932 pmol cyclic AMP hydrolyzed/mg protein per min in the presence of Ca^{2+} and protein activator.

Tissue fractionation. Cattle eyes were purchased from a local slaughterhouse. Retinas and lenses were dissected out under normal lighting conditions and were homogenized in a buffer containing 0.32 M sucrose, 1.5 mM $MgSO_4$ and 40 mM Tris · HCl (pH 7.6). The subcellular fractionation of the retina was performed essentially according to De Robertis et al. [16]. The fractions of lens were prepared by centrifugation at 105000 × *g* for 1 h. The fractions derived from both the retina and lens were dialyzed against 20 mM Tris · HCl (pH 7.5), (1 : 400, v/v) with three changes.

Preparation of bovine retina rod outer segment. Bovine rod outer segment was prepared according to the method of Wong et al. [17]. The precipitate of rod outer segment after sucrose density gradient centrifugation was then homogenized in 20 mM Tris · HCl/0.9% NaCl, incubated for 2 h at 4°C and centrifuged at 12000 × *g* for 30 min. The extraction procedure using 20 mM Tris · HCl/0.9% NaCl as solvent was repeated twice more and the supernatants were combined and dialyzed against 20 mM Tris · HCl (pH 7.5) (1 : 400, v/v) with three changes.

Assay of phosphodiesterase. Phosphodiesterase activity was measured using an anion exchange resin [18]. The assay mixture contained 40 mM Tris · HCl

(pH 8.0)/3 mM $MgSO_4$/50 μM $CaCl_2$/1 μM unlabeled and ^{3}H-labeled cyclic AMP/an appropriate amount of enzyme, in total volume of 100 μl. After 10 min incubation at 30°C, the reaction was terminated by placing the tubes in boiling water for 45 s. 50 μg snake venom was added to convert 5'-AMP to adenosine. At the end of 10 min incubation at 30°C, 1 ml IRP-58 resin was added to terminate the reaction. Denatured proteins and unreacted substrate were precipitated with resin by centrifugation, and a fraction of the supernatant which contained [^{3}H]adenosine was counted by liquid scintillation spectrometry.

Assay of activator. The activator was assayed by testing its ability to stimulate the activity of activator-deficient enzyme (0.68 μg). The procedure was the same as the assay for phosphodiesterase except that the boiled samples were included as the source of activator. One unit of activator was defined as the amount required to give half-maximum stimulation of activator-deficient enzyme.

Measurement of protein. Protein was determined according to Lowry et al. [19] with bovine serum albumin as standard.

Results

Phosphodiesterase activity of bovine retina, bovine rod outer segment and bovine lens is distributed in both particulate and soluble fractions. The subcellular distribution of retinal enzyme activity was assayed at a low substrate concentration (1 μM). These experiments showed that the rate of hydrolysis of cylcic GMP was 1.6—11 times higher than that of cylic AMP in all fractions (Table I). The highest total cyclic AMP phosphodiesterase (34%) was found in

TABLE I

SUBCELLULAR DISTRIBUTION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY (PDE) IN BOVINE RETINA

A fresh homogenate diluted to 10% in 0.32 M sucrose, 1.5 mM $MgCl_2$ and 40 mM Tris · HCl (pH 7.6) was centrifuged for 10 min at 900 × *g* at 4°C. The precipitate after being twice washed and centrifuged was designated the nuclear fraction. The supernatant was pooled and centrifuged at 11 500 × *g* for 20 min. The resultant pellet was washed and centrifuged. This precipitate was termed the mitochondrial fraction. The supernatant was centrifuged at 105 000 × *g* for 60 min. The precipitate was denoted the microsomal fraction and the soluble fluid as the supernatant fraction. All fractions were dialyzed against 20 mM Tris · HCl (pH 7.5, 1 : 400, v/v) with three changes. The activity of phosphodiesterase was determined in triplicates. The percent total activity was calculated form the total activity in each fraction divided by the sum of total activity of all fractions. The recovery of activity was about 80% for both cyclic AMP and cyclic GMP phosphodiesterase.

Fraction	Protein (mg)	CyclicAMP PDE		CyclicGMP PDE		CyclicGMP PDE/ cyclicAMP PDE
		pmol/mg per min	% Total activity	pmol/mg per min	% Total activity	
Homogenate		563		5090		9
Nuclear	10.4	261	19	2950	60	11
Mitochondria	6.7	346	16	692	9	2
Microsomes	5.7	780	31	1230	14	1.6
Supernatant	16.9	290	34	520	17	1.8

TABLE II

DISTRIBUTION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY (PDE) IN BOVINE ROD OUTER SEGMENT

Bovine rod outer segment was prepared according to the method of Wong et al. [17] and the precipitate was separated form the soluble fraction by centrifugation at 12 000 × *g* for 30 min. The assay conditions for the phosphodiesterase activity were the same as in Table I. The percent total activity was calculated from the total activity in precipitation supernatant divided by the sum of total activity of precipitate and supernatant.

Fraction	Protein (mg)	CyclicAMP PDE		CyclicGMP PDE		CyclicGMP PDE/ cyclicAMP
		pmol/mg per min	% Total activity	pmol/mg per min	% Total activity	
Precipitate	8.1	195	96	2344	99.7	12
Supernatant	2.8	26	4	21	0.3	0.8

the 105000 × *g* supernatant, while 60% of cyclic GMP phosphodiesterase activity was in the nuclear fraction.

After density gradient centrifugation in sucrose, the purified bovine rod outer segment was separated into soluble and membrane-bound fractions by repeating extractions with 20 mM Tris · HCl/0.9% NaCl. The phosphodiesterase activities in both particulate and supernatant fractions were determined. The soluble fraction contained very little enzyme activity, as shown in Table II. Almost all activity remained in the particulate phase with specific activity of cyclic GMP phosphodiesterase 12 times higher than that of cyclic AMP phosphodiesterase.

Phosphodiesterase activity of the bovine lens exists in still another distribution pattern which is different from that of retina and rod outer segment. As shown in Table III, the 105000 × *g* supernatant has 73 and 96% of the total cyclic AMP and cyclic GMP phosphodiesterase activity of this structure, respectively. The hydrolysis of cyclic AMP was 17 times faster than that of cyclic GMP in the supernatant. In the particulate fraction of bovine lens, the difference in hydrolysis was even greater, with cyclic AMP hydrolysis 169 times faster than that for cyclic GMP hydrolysis.

TABLE III

DISTRIBUTION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY (PDE) IN BOVINE LENS

The fractions of the bovine lens were preapred by centrifugation at 105 000 × *g* for 1 h and were dialyzed against 20 mM Tris · HCl (pH 7.5, 1 : 400, v/v) with three changes. The enzyme activity was determined in triplicate. The calculation of the percent total activity is the same as in Table II.

Fraction	Protein (mg)	CyclicAMP PDE		CyclicGMP PDE		CyclicGMP PDE/ cyclicAMP
		pmol/mg per min	% Total activity	pmol/mg per min	% Total activity	
Homogenate		2.3		0.104		22
Precipitate	260	4.4	27.5	0.026	3.6	169
Supernatant	1258	2.4	72.5	0.143	96.4	17

TABLE IV

EFFECT OF EGTA ON CYCLIC AMP PHOSPHODIESTERASE ACTIVITY (PDE) AND DISTRIBUTION OF PROTEIN ACTIVATOR ACTIVITY OF CYCLIC AMP PHOSPHODIESTERASE IN BOVINE RETINA, ROD OUTER SEGMENT AND LENS

Enzyme activity was assay with 1 μM cyclic AMP in the presence of 200 μM EGTA. Percent of control was the ratio of enzyme activity measured in the presence of EGTA to the enzyme activity obtained in the absence of EGTA. Protein activator activity of cyclic AMP phosphodiesterase was assayed. The calculation of the percent total activity is the same as in Table I. The recovery in activator activity was about 90%.

Fraction	PDE + EGTA (% of control)	Protein activator	
		unit/mg	% Total activity
Retina			
Homogenate	90	167	
Nuclear	100	172	24
Mitochondria	97	170	15
Microsomes	100	256	19
Supernatant	79	185	42
Rod out segment			
Precipitate	100	100	83
Supernatant	100	61	17
Lens			
Homogenate	99	11	
Precipitate	100	10	14
Supernatant	96	13	86

Both Ca^{2+}-dependent and Ca^{2+}-independent forms of phosphodiesterase have been demonstrated in many tissues [20—23]. We have examined the effect of the chelating agent EGTA on the cyclic AMP phosphodiesterase activity of eye tissues including bovine retina and bovine lens. Cyclic AMP phosphodiesterase activity in the homogenate and in the 105000 $\times g$ supernatant of bovine retina have 10 and 20% Ca^{2+}-dependent form, respectively (Table IV). Other subcellular fractions of bovine retina, the precipitate and supernatant of bovine rod outer segment and fractions derived from bovine lens, showed very little effect by the addition of 200 μM EGTA. The results indicate that cyclic AMP phosphodiesterase activity exists as a Ca^{2+}-independent form in most fractions of bovine retina, in bovine rod outer segment and in bovine lens, as judged by the minimal depression of enzyme activity due to the chelation of Ca^{2+} by EGTA.

Since the Ca^{2+}-dependent form of cyclic AMP phosphodiesterase was found only in the supernatant fraction of the retina, the question is raised as to the presence of phosphodiesterase protein activator in the retinal subcellular fractions, rod outer segment and in bovine lens. We have found that protein activator exists in all fractions (Table IV). The highest specific activity of the activator was obtained in the retinal microsomal fraction and the lowest was in the lens. These results suggest that the protein activator may have functions other than an activator of cyclic AMP phosphodiesterase.

Phosphodiesterases from many tissues exhibit kinetic complexity, as indicated by non-linear Lineweaver-Burk plots [24—28]. This kinetic compleixity has been attributed to the existence of multiple enzymes with different affini-

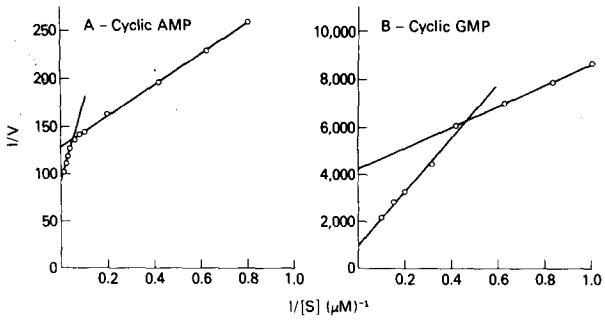

Fig. 1. Kinetic analysis of cyclic nucleotide hydrolysis by the lens supernatant. (A) Cyclic AMP hydrolysis; (B) cyclic GMP hydrolysis. The supernatant fraction of bovine lens was prepared at 105000 × *g* for 60 min in a buffer containing 0.32 M sucrose/1.5 mM $MgSO_4$/40 mM Tris · HCl (pH 7.6). The supernatant was dialyzed against 20 mM Tris · HCl (pH 7.5, 1 : 400, v/v) with three changes and was then assayed for phosphodiesterase activity at 1—80 μM substrate.

ties for substrates. Kinetic analysis revealed similar complexity in phosphodiesterase from the 105000 × *g* supernatant of bovine lens (Fig. 1). Michaelis constant, K_m, for cyclic nucleotides was calculated from the Lineweaver-Burk plots, with substrate concentrations from 1 to 80 μM. The apparent K_m values for both cyclic AMP ($1.3 \cdot 10^{-6}$ and $9.2 \cdot 10^{-6}$ M) and cyclic GMP ($1.04 \cdot 10^{-6}$ and $12.2 \cdot 10^{-6}$ M) are very similar. Although the cyclic nucleotide phosphodiesterase and the protein activator of cyclic AMP phosphodiesterase are present in the bovine lens, the physiological functions of cyclic nucleotide system in th lens still remains unknown.

Discussion

When phosphodiesterase activity was measured at substrate concentrations close to physiological levels, the hydrolysis of cyclic GMP was faster than that for cyclic AMP in subcellular fractions derived from the bovine retina. Chader et al. [9] have reported similar findings with 5 μM substrate concentration, although the enzyme specific activity and the ratio of cyclic GMP phosphodiesterase to cyclic AMP phosphodiesterase appeared to be different in some fractions. The differences could be due to the existence of different kinetic forms of phosphodiesterase which are active at different substrate levels.

In bovine rod outer segment, the major portion of hydrolytic activity for both cyclic AMP and cyclic GMP was found in the membrane-associated phosphodiesterase. The high activity of cyclic GMP phosphodiesterase found in the precipitate of the rot outer segment was consistent with reports from other laboratories [6—11]. The reason for the lower phosphodiesterase activity in the supernatant may be due to the denaturation of the enzyme caused by the repeated extraction. It may also be due to the inability to extract the enzyme and the protein activator from the rod outer segment precipitate derived from a sucrose density gradient centrifugation. However, 17% of the activator activity detected in the supernatant (Table IV) may indicate that the protein activator has weaker affinity than that of phosphodiestnase to the rod outer segment membrane.

The hydrolysis of cyclic AMP is much faster than that of cyclic GMP by the bovine lens phosphodiesterase, especially for the membrane-bound enzyme. This hydrolysis pattern is inverse to that of the retina and rod outer segment. As seen in Table III, only 4% of cyclic GMP phosphodiesterase was found in the precipitate, whereas 28% of the cyclic AMP phosphodiesterase was found in the precipitate. This, together with the findings that the percent activity of cyclic AMP and cyclic GMP phosphodiesterase did not show parallel relationship in the fractions obtained from the bovine retina and from the rod outer segment suggests that there might be separate enzymes for the hydrolysis of cyclic AMP and cyclic GMP.

Cyclic AMP phosphodiesterase has at least two different molecular forms: the Ca^{2+} and protein activator dependent form and the Ca^{2+}-independent form. As shown in Table IV, the cyclic AMP phosphodiesterase activity is not influenced by the addition of EGTA in any fractions except in the supernatant of the retina. The result suggests that the phosphodiesterase is a "native" Ca^{2+}-independent form or a "modified" Ca^{2+}-independent form derived from a Ca^{2+}-dependent form as the result of partial digestion by proteolytic enzymes. Cheung [29] and Miki and Yoshida [30] reported that the Ca^{2+}-dependent form of the brain phosphodiesterase after the trypsin treatment was no longer sensitive to EGTA. The conversion of a Ca^{2+}-dependent form of phosphodiesterase to a Ca^{2+}-independent form by proteolytic digestion may explain the fact that the protein activator of cyclic AMP phosphodiesterase existed in all fractions. Alternatively, the protein activator may have functions other than the activator of cyclic AMP phosphodiesterase. Brostrom et al. and Cheung et al. [31,32] reported that protein activator also stimulates Lubrol-solubilized brain adenylate cyclase. Close homology in structure has been demonstrated between activator from bovine brain and beef heart and troponin C from rabbit skeletal muscle, the latter being a protein which regulates actomyosin ATPase [33]. Recently, it has been reported that the protein activator resembles the red blood cell cytoplasmic activator of (Ca^{2+} + Mg^{2+})-ATPase [34,35]. The range of functions of these small molecular weight "activator" proteins remains to be identified.

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