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A KINETIC ANALYSIS OF THE CYCLIC NUCLEOTIDE PHOSPHODIESTERASE REGULATION BY THE ENDOGENOUS PROTEIN ACTIVATOR

A STUDY OF RAT BRAIN AND FROG SYMPATHETIC CHAIN

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Summary

The effect of the endogenous protein activator on the kinetic characteristics of a highly purified, activator-deficient rat brain phosphodiesterase (EC 3 1 4.-) was studied This enzyme preparation has only a high K_m for cyclic AMP and a low K_m for cyclic GMP In the presence of 20 μM Ca^{2+}, saturating concentrations of the activator decreased the K_m of this enzyme for cyclic AMP from 350 μM to about 80 μM, without changing the V The phosphodiesterase activator did not change the K_m of phosphodiesterase for cyclic GMP, however, a moderate increase of V was seen The activator lacks species specificity, the activator isolated from the bullfrog sympathetic chain produced the same qualitative and comparable quantitative changes in the kinetic properties of the purified rat brain phosphodiesterase

Cyclic GMP is a potent competitive inhibitor of the phosphodiesterase activation by the activator (K_i = 1 8 μM), using cyclic AMP as a substrate Cyclic AMP inhibits slightly the hydrolysis of cyclic GMP by phosphodiesterase in the presence of activator (K_i = 155 μM) only

Introduction

Cyclic 3′,5′-nucleotide phosphodiesterase (EC 3 1 4 -) is the only enzyme that catabolizes 3′,5′-cyclic adenosine monophosphate (cyclic AMP) and 3′,5′-cyclic guanosine monophosphate (cyclic GMP) in the central nervous system and in other tissues, there are various molecular forms of phosphodiesterase present in any given tissue [1—13] Some forms of phosphodiesterase require specific ions, others require an endogenous protein activator [5,7,13—16] The

activator regulates various cyclic AMP phosphodiesterase preparations by decreasing the K_m of the enzyme for cyclic AMP [7], by increasing the V of the enzyme [15], or by changing both kinetic constants [17] Teo et al [18] suggested that the type of action elicited by the activator on the kinetic properties of phosphodiesterase depends upon its concentration In low concentrations, the activator increases the V of an activator-deficient phosphodiesterase when cyclic AMP is used as a substrate, whereas in high concentrations the activator only decreases the K_m Since phosphodiesterase homogeneity and purity varied in these studies, it is difficult to offer a unified explanation of the interaction of phosphodiesterase and the activator at the molecular level However, it is apparent that those forms of phosphodiesterase which are activator-sensitive have a high K_m for cyclic AMP and catalyze the hydrolysis of both cyclic AMP and cyclic GMP [7,11]

In the present paper we report investigations on the mechanisms whereby the activator changes the kinetic parameters of a highly purified phosphodiesterase prepared from rat brain, which is termed phosphodiesterase Peak II, to facilitate direct reference to our previous work [7]. This preparation of phosphodiesterase Peak II gives a single band on analytical gel electrophoresis and shows a classical type of Michaelis-Menten kinetics over a wide range of cyclic AMP concentrations Thus, the enzyme has simple kinetic characteristics and a high K_m for cyclic AMP In this study we found that the activator decreases the K_m of phosphodiesterase Peak II for cyclic AMP and increases the V for cyclic GMP. In addition, the effect of cyclic GMP on the hydrolysis of cyclic AMP by phosphodiesterase Peak II, and vice versa, was studied using both the activator-deficient phosphodiesterase and the activated enzyme

Methods

Materials Brains from Sprague—Dawley rats obtained from Zivic Miller (Allison Park, Pa) were used for the purification of phosphodiesterase Peak II [7] Sympathetic ganglia were isolated from bullfrogs (*Rana catesbeiana*) purchased from E G Hoffman (Oshkosh, Mich) ^{3}H-labeled cyclic AMP (specific radioactivity 37.7 Ci/mmol) and ^{3}H-labeled cyclic GMP (specific radioactivity 4 8 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, Mass) Before use, ^{3}H-labeled cyclic AMP was purified by column chromatography on Dowex 1 (X2, 200—400 mesh, Cl^- form) and ^{3}H-labeled cyclic AMP on Dowex 50 (X8, 200—400 mesh, H^+ form) Unlabeled cyclic AMP and cyclic GMP were purchased from Sigma Chemical Co (St Louis, Mo) The compounds used for preparation of polyacrylamide gels (acrylamide, *N,N*-methylenebisacrylamide, *N,N,N',N'*-tetramethylenediamine and $(NH_4)_2 S_2 O_8$) were obtained from Eastman Organic Chemicals (Rochester, N Y) Firefly luciferin-luciferase was obtained from E I Dupont de Nemours and Co (Wilmington, Del) The two sources of 5'-nucleotidase (EC 3.1 3 5) were the venom of *Ophiophagus hannah* and a purified 5'-nucleotidase both from Sigma Chemical Co (St Louis, Mo)

Isolation of phosphodiesterase Peak II The activator-deficient phosphodiesterase Peak II was isolated according to Uzunov and Weiss [7] Male Sprague-Dawley rats (200—250 g) were killed by decapitation The brains were rapidly removed, placed on ice and freed from dura mater tissues and blood vessels

These brains were minced with scissors, homogenized in 0 32 M sucrose, sonicated for 5 min and centrifuged at 100 000 × *g* for 1 h The soluble supernatant was used for phosphodiesterase Peak II purification by preparative polyacrylamide column electrophoresis [7] The apparatus used was manufactured by Shandon Scientific Co (Phila , Pa) The column (11 cm in height) was prepared with 7 5% acrylamide gel, polymerized in the presence of $(NH_4)_2 S_2 O_8$ and 0 37 M Tris HCl buffer, pH 9 2 To remove the polymerization catalyst the column was washed for 1 h with this Tris buffer by applying a current of 20 mA The 100 000 × *g* supernatant of the brain homogenates was loaded on this column and chromatographed for 1 h with a current of 25 mA using Tris/glycine (0 08 M), pH 8 3 Then the current intensity was increased to 60 mA and kept at this level for 20 h The proteins were eluted from the elution chamber with a constant stream of 0 43 M Tris/acetic acid buffer, pH 7 6 200 1-ml fractions were assayed for phosphodiesterase activity both in the presence and in the absence of added endogenous activator Phosphodiesterase Peak II was eluted from the column in the 76 to 86 fractions These fractions were pooled together and frozen in small aliquots which were stored at −80° C for use at a later time An aliquot was used to ascertain the enzyme purity by rechromatography on analytical polyacrylamide gel electrophoresis This procedure yields a single protein peak, which contains all the phosphodiesterase activity This preparation has a specific activity of 3200 pmol cyclic AMP hydrolyzed/μg protein per min The specific activity of whole brain homogenate phosphodiesterase was 20 pmol/μg protein per min, giving a 160-fold purification

The phosphodiesterase activity of various eluate fractions was assayed by the method of Weiss et al [19] Briefly, this assay is based on the stoichiometric conversion of 5′-AMP, the product of phosphodiesterase activity, to ATP by the addition of an excess myokinase and pyruvate kinase The ATP formed was assayed with a luminescence biometer (Dupont and Co , Wilmington, Del) by measuring the light generated when firefly luciferin-luciferase is added

The kinetics of the purified phosphodiesterase Peak II were studied by the isotopic method of Filburn and Karn [20], using either cyclic AMP or cyclic GMP as a substrate A standard mixture in a final volume of 100 μl contained varying concentrations of substrate (120 000—130 000 cpm ^3H), 1 mM Mg^{2+}, 20 μM Ca^{2+}, 0 6 mM dithiothreitol, 32 mM Tris HCl (pH 7 5) and purified enzyme The mixtures were incubated at 37° C, during our standard incubation time (5 min) no more than 20% of any given substrate concentration was hydrolyzed The reaction velocity was linear up to 0 2 μg protein per μl The reaction was stopped by placing the test tubes in boiling water for 1 min Then 50 μg of *Ophiophagus hannah* venom or 4 μg of purified 5′-nucleotidase were added to the mixtures and the samples were reincubated for 30 min at 37° C This treatment yields quantitative conversion of the 5′-nucleotides to the corresponding nucleosides The reaction was stopped by the addition of 400 μl of 0 1 M ammonium acetate buffer, pH 4 Adenosine or guanosine were separated from the enzymatic mixture on neutral aluminum oxide columns which were previously equilibrated with the ammonium acetate buffer The effluent was collected and the nucleosides were eluted by adding 1 5 ml of ammonium acetate buffer, pH 4 The combined effluents and eluates were added to aqua-

sol and counted in a Beckman liquid scintillation spectrometer. Counting efficiency for ^3H was 18%

Preparation of phosphodiesterase activator The phosphodiesterase activator was isolated from rat brain using the method of Lin et al. [21] with a slight modification The procedure involved $(NH_4)_2SO_4$ precipitation, heat treatment, DEAE-cellulose chromatography and preparative polyacrylamide column gel electrophoresis The activator was prepared from the bullfrog sympathetic chain according to a procedure previously described [22]

Results

(A) Effect of phosphodiesterase activator on the hydrolysis of cyclic AMP by phosphodiesterase Peak II The phosphodiesterase activity from rat brain included in Peak II, isolated by preparative polyacrylamide gel electrophoresis, represents a major proportion (68%) of the brain phosphodiesterase activity when cyclic AMP is the substrate. Reciprocal plots of phosphodiesterase Peak II activity against a wide range of cyclic AMP concentrations exhibit only one apparent K_m (about 350 μM). The reciprocal plots of phosphodiesterase Peak II activity in the presence and in the absence of the activator are presented in Fig 1 The maximal decrease in the K_m of the enzyme was elicited by 20 μg of activator, a greater concentration of this protein fails to show an additional increase in the affinity of phosphodiesterase Peak II for cyclic AMP With 20 μg of activator, the maximal velocity of the phosphodiesterase activity remained unchanged (Fig 1), while the K_m for cyclic AMP decreased by more than 4-fold (from 350 to about 80 μM) Saturating concentrations of activator produced no more than a 30% increase of the V of phosphodiesterase Peak II, using cyclic AMP as a substrate.

(B) Effect of cyclic GMP on the hydrolysis of cyclic AMP by phosphodiesterase Peak II These studies were performed to ascertain whether cyclic GMP changes the hydrolysis of cyclic AMP by both activator-deficient and activator-

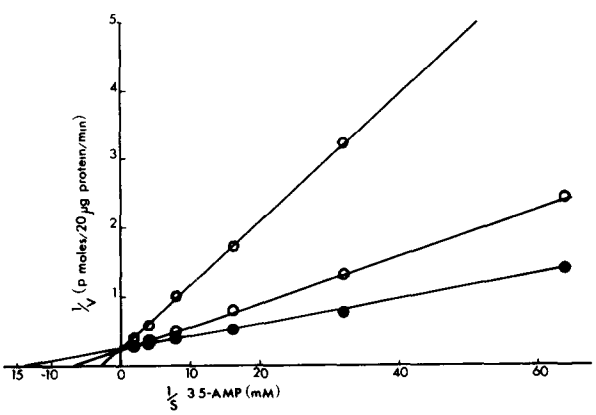

Fig 1 Double reciprocal plot of the initial velocity of phosphodiesterase Peak II versus 3′,5′-AMP (cyclic AMP) concentration in the presence of subsaturating and saturating concentrations of the activator The conditions of the assay are described under Methods ◇———◇, no addition, ○———○, 10 μg of activator, ●———●, 20 μg of activator

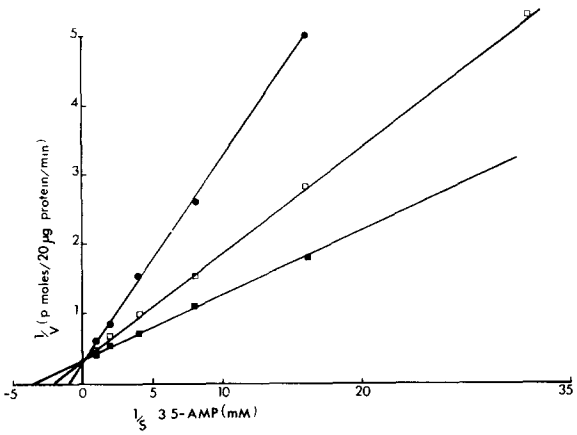

Fig 2 Inhibition by cyclic GMP of activator-deficient phosphodiesterase Peak II ■———■, no cyclic GMP, □———□, 1 10^{-5} M cyclic GMP, ●———●, 2 10^{-5} M cyclic GMP

saturated phosphodiesterase Peak II The results are presented in Figs 2 and 3 In both cases, cyclic GMP is a potent competitive inhibitor of cyclic AMP, the K_i values for the activator-deficient and activator-saturated phosphodiesterase were 2.0 and 1.8 μM, respectively

(C) Effect of phosphodiesterase activator on the hydrolysis of cyclic GMP by phosphodiesterase Peak II The hydrolysis of cyclic GMP by phosphodiesterase Peak II in the presence or in the absence of the activator is presented in Fig 4 The activator-deficient phosphodiesterase Peak II has a high affinity for cyclic GMP and hydrolyzes this mononucleotide with a V greater than that displayed for cyclic AMP The apparent K_m of phosphodiesterase Peak II for cyclic GMP is 5—9 μM The addition of saturating concentrations of the activator did not change the K_m of phosphodiesterase Peak II for cyclic GMP but it increased its V by about 2-fold. These kinetic constants were characterized using a wide range of cyclic GMP and activator concentrations The effects of activator from rat brain on the hydrolysis of cyclic AMP and cyclic GMP by phosphodiesterase peak II are summarized in Table I

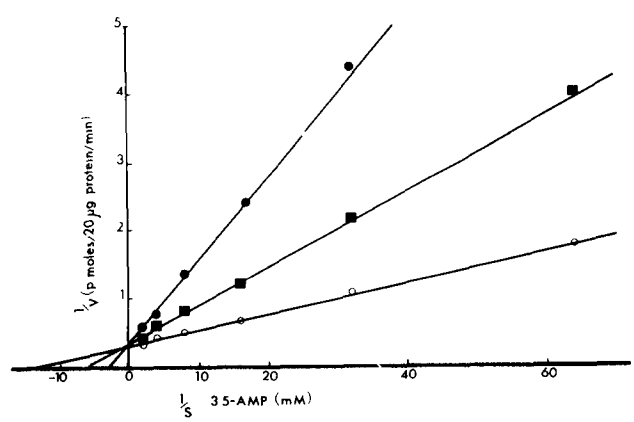

Fig 3 Inhibition of activator-saturated phosphodiesterase Peak II by cyclic GMP The concentrations of cyclic GMP were ○———○, none, ■———■, 1 10^{-5} M, and ●———●, 2 10^{-5} M

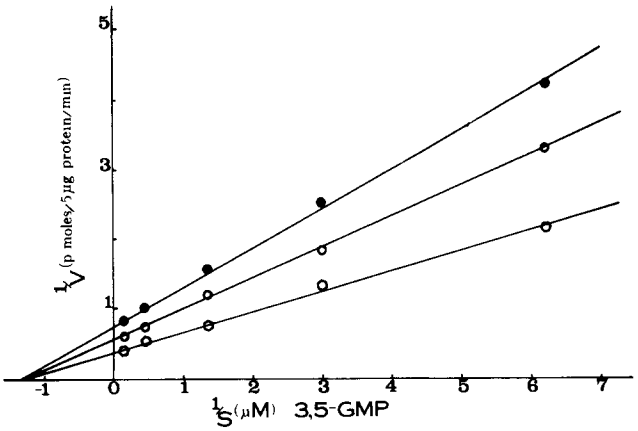

Fig 4 Double reciprocal plot of the initial velocity of phosphodiesterase Peak II versus 3',5'-GMP (cyclic GMP) in the presence of subsaturating and saturating concentrations of the activator The concentrations of activator were ●——●, none, ○——○, 10 μg, and ○——○, 20 μg

(D) Effect of cyclic AMP on the hydrolysis of cyclic GMP by phosphodiesterase Peak II in the presence and in the absence of phosphodiesterase activator Cyclic AMP ($5 \cdot 10^{-5}$ M) did not change the hydrolysis of cyclic GMP by the activator-deficient phosphodiesterase Peak II. In the presence of saturating concentrations of endogenous activator, however, cyclic AMP inhibited the enzyme activity This inhibition appeared to be of a competitive type with a K_i value of 155 μM

(E) Experiments with phosphodiesterase activator from bullfrog sympathetic chain The activator isolated from the bullfrog sympathetic chain produced the same qualitative and comparable quantitative changes in the kinetic properties of purified phosphodiesterase Peak II as those elicited by the activator isolated from rat brain It decreased the K_m for cyclic AMP by more than 3-fold and increased the V for cyclic GMP by about 2-fold.

Discussion

The results from our study offer a basis for understanding the possible role of the phosphodiesterase activator in the regulation of cyclic AMP and cyclic

TABLE I

EFFECT OF THE ENDOGENOUS PROTEIN ACTIVATOR FROM RAT BRAIN ON PHOSPHODIESTERASE PEAK II

The phosphodiesterase activator was isolated as described in Methods The activator (10 μg protein) was added to activator-deficient phosphodiesterase using either cyclic AMP or cyclic GMP as a substrate Each value was calculated on the basis of five enzyme determinations

Addition	K_m (μM)		V (nmol/ml eluate per min)	
	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
None	350 ± 15	9 ± 0 3	20 ± 1 5	7 5 ± 0 5
Phosphodiesterase activator	80 ± 0 4	9 ± 0 5	19 ± 1 3	18 2 ± 2 0

GMP content in the cell Using purified phosphodiesterase Peak II from rat brain which has high K_m for cyclic AMP (350 μM) and low K_m for cyclic GMP (5—9 μM) it was found that the activator lowers the apparent K_m of this enzyme for cyclic AMP by more than 4-fold No changes in the apparent K_m of phosphodiesterase Peak II for cyclic GMP was found A wide range of both subsaturating and saturating concentrations of the activator did not change the V of phosphodiesterase Peak II activity significantly, using cyclic AMP as a substrate The V increased by about 30% whereas the apparent K_m of phosphodiesterase Peak II for cyclic AMP decreased to a level approximately one-fourth of that observed in the absence of the activator Thus the activator increases the affinity of the Peak II phosphodiesterase for cyclic AMP four-fold without changing the V The V of phosphodiesterase Peak II is several-fold higher than that of the other molecular forms of phosphodiesterase, including those forms that have low K_m for cyclic AMP [22] In the natural form, phosphodiesterase Peak II (K_m 350 μM) is highly unlikely to hydrolyze the concentration of cyclic AMP normally present in the cell (10^{-6} M) This inference may be true also when the intracellular concentrations of cyclic AMP doubles However, when phosphodiesterase Peak II is saturated with activator, the enzyme affinity for cyclic AMP is within the range of the tissue content The high hydrolytic capacity of the phosphodiesterase Peak II is eminently suitable for a rapid catabolism of cyclic AMP when the tissue content is increased following adenylate cyclase stimulation by hormones and neurotransmitters This consideration is in line with previous reports that the activity of the activator increases in the cytoplasm of several tissues when cyclic AMP content is elevated through a transsynaptic activation of adenylate cyclase [23—25] It has been suggested previously that the high K_m phosphodiesterase is the enzyme that operates when the concentrations of cyclic AMP are high and most probably protects the cell from exceptionally high accumulation of this second messenger [26] However, without the activator participation, phosphodiesterase Peak II would have almost no role in modulating the hydrolysis of cyclic AMP unless the cyclic nucleotide concentrations were at least 100-fold greater than normal

Previous kinetic studies of the effect of the endogenous protein activator on the high K_m phosphodiesterase revealed changes of the kinetic characteristics of this enzyme, which are similar to those described in this report [17,18] A comparison of our data with those of Appleman and Terasaki [27] shows a number of similarities their phosphodiesterase D-I, obtained essentially by a single-step purification has a high K_m for cyclic AMP (more than 200 μM), a low K_m for cyclic GMP and a very high V when cyclic AMP is the substrate. Our phosphodiesterase Peak II has almost identical characteristics When phosphodiesterase D-I is saturated with the activator, the V increases by 30% but the K_m decreases by only 60% However, the K_m of phosphodiesterase Peak II can be lowered by more than 400% in piesence of activator Probably the DEAE-cellulose column used by Appleman and Terasaki [27] fails to produce a separation of the activator from phosphodiesterase as complete as that obtained with polyacrylamide gel electrophoresis The results of the present study are at variance with a report by Weiss et al [28] concerning the effects of the activator on phosphodiesterase Peak II The preparation of the enzyme was virtually the same, however, Weiss et al [28] found that the activator increases

the V but does not decrease the K_m of phosphodiesterase Peak II for cyclic AMP The only possible explanation for this discrepancy is the difference in the methods used for determining phosphodiesterase activity. The very sensitive method of Weiss et al. [19] to assay phosphodiesterase is based on the quantitative conversion of cyclic AMP into ATP and consecutive light formation by using a cycle of four enzyme reactions Perhaps this otherwise useful method for phosphodiesterase assay is not the method of choice to study phosphodiesterase kinetics

The finding that the activator isolated from bullfrog sympathetic ganglia also decreases the K_m of phosphodiesterase Peak II for cyclic AMP without changing the V suggests that activator present in tissues of different species may act on phosphodiesterase Peak II in a similar manner Therefore, one might infer that the activator regulates phosphodiesterase with a mechanism that lacks both tissue and species specificity and is dependent on micromolar Ca^{2+} concentrations [5,21]

Our studies confirm that cyclic GMP inhibits cyclic AMP hydrolysis by both activator-deficient and activator-saturated phosphodiesterase Peak II [27,29,30] The K_i values of cyclic GMP for the inhibition of cyclic AMP hydrolysis are very close to the K_m of the enzyme for cyclic GMP. This finding raises the possibility that phosphodiesterase activity could be regulated by cyclic GMP concentrations in tissues where the cyclic GMP content is relatively high (i e. cerebellum [31]). Since cyclic GMP content in cerebellum [31,32] and in adrenal medulla [33] can increase by several-fold without a corresponding change in cyclic AMP content, we can surmise that the compartmentation of the two nucleotides may prevent their direct interaction Other explanations could be postulated but it is clear that the regulatory function of the activator cannot be understood completely until we know the molecular nature of the changes that occur in this protein when the tissue content of cyclic AMP or cyclic GMP increases. The development of inhibition of the activated phosphodiesterase Peak II by cyclic AMP when cyclic GMP is used as a substrate is within the lines of the expectations Obviously, the appearance of inhibition in the presence of saturating concentrations of activator reflects the decrease of the K_m of phosphodiesterase Peak II for cyclic AMP

In conclusion, this study shows that the activator can lower the K_m for cyclic AMP of a phosphodiesterase with high K_m for this nucleotide This finding and the sensitivity of phosphodiesterase Peak II for activator activation supports the view that the kinetic properties of phosphodiesterase are regulated in vivo by the activator [21] and that an increase in activator activity reflects a previous increment of second messengers responses It remains to be elucidated whether the activator activity increase reflects a new synthesis of activator or a release of activator from a binding site in tissues. Preliminary experiments being conducted in our laboratory appear to support the latter view

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