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RECONSIDERATION OF THE MULTIPLE CYCLIC NUCLEOTIDE PHOSPHODIESTERASES IN BOVINE HEART

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SUMMARY: DEAE-cellulose chromatography demonstrated the presence of three peaks of cyclic nucleotide phosphodiesterase activity in the hearts of cattle during the summer and only two peaks during exposure to freezing temperatures. The hydrolysis of 10^{-6}M cyclic AMP by peak II, the variable activity, was stimulated 160% by 10^{-6}M cyclic GMP and was inhibited by chelation of Ca^{2+}. Peak II activity was not a distinct enzyme but rather a mixture of activator-dependent phosphodiesterase, phosphodiesterase activator and type II cyclic AMP-dependent protein kinase.

INTRODUCTION: Multiple forms of cyclic nucleotide phosphodiesterase have been identified in extracts of all mammalian tissues examined (1,2). These forms differ in their kinetic behavior, substrate specificity and response to various effectors and seem to exist in different ratios in various cell types. Most mammalian tissues except human lung (3), guinea pig lung (4) and rat erythrocytes (5) possess a cyclic nucleotide phosphodiesterase which is stimulated by a heat-stable activator protein in the presence of Ca^{2+} (6,7). At low substrate levels this enzyme hydrolyzes 2-4 fold more cyclic GMP than cyclic AMP. The hydrolysis of 10^{-6}M cyclic AMP by a second type of phosphodiesterase is stimulated several fold by the addition of 10^{-6}M cyclic GMP (8,9). At low substrate levels this enzyme hydrolyzes 1.5-3 fold more cyclic GMP than cyclic AMP. A third enzyme is rather specific for cyclic AMP (1,8). Bovine heart has been reported to have these three enzyme activities (10) however the present report suggests that the second type of enzyme activity isolated from this tissue and possibly other tissues by DEAE-cellulose chromatography may not represent a distinct enzyme but rather a mixture of activator-dependent phosphodiesterase and several regulatory proteins.

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MATERIALS AND METHODS: Cyclic AMP, cyclic GMP, EGTA and Crotalus atrox venom were purchased from Sigma. Cyclic [^{3}H]AMP and cyclic [^{3}H]GMP were from Amersham/Searle and [γ-^{32}P]ATP was from New England Nuclear. Bovine hearts were obtained from a local slaughterhouse during summer and early fall.

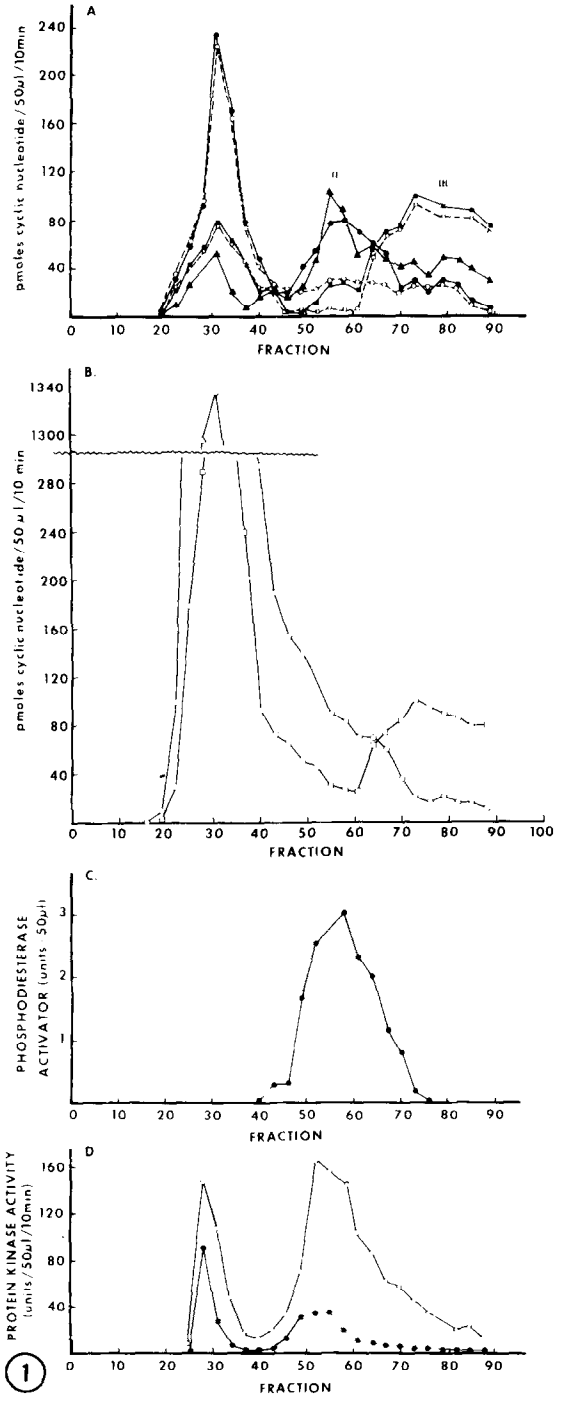
Phosphodiesterase activity was assayed by a procedure adapted from Russell et al. (8). An appropriate aliquot of enzyme was incubated in 40 mM Tris Cl, pH 7.4 and 5 mM $MgCl_2$ containing 10^{-6}M cyclic [^{3}H]AMP or cyclic [^{3}H]GMP (50,000 counts per min) in a total volume of 1 ml. Alterations of this procedure are as indicated in the legends to the figures. After 10 min at 30°C, the reaction was terminated by boiling for 3 min, and the sample processed as described (2). The amount of phosphodiesterase used was adjusted so that no more than 15% of the cyclic nucleotide was hydrolyzed during the incubation.

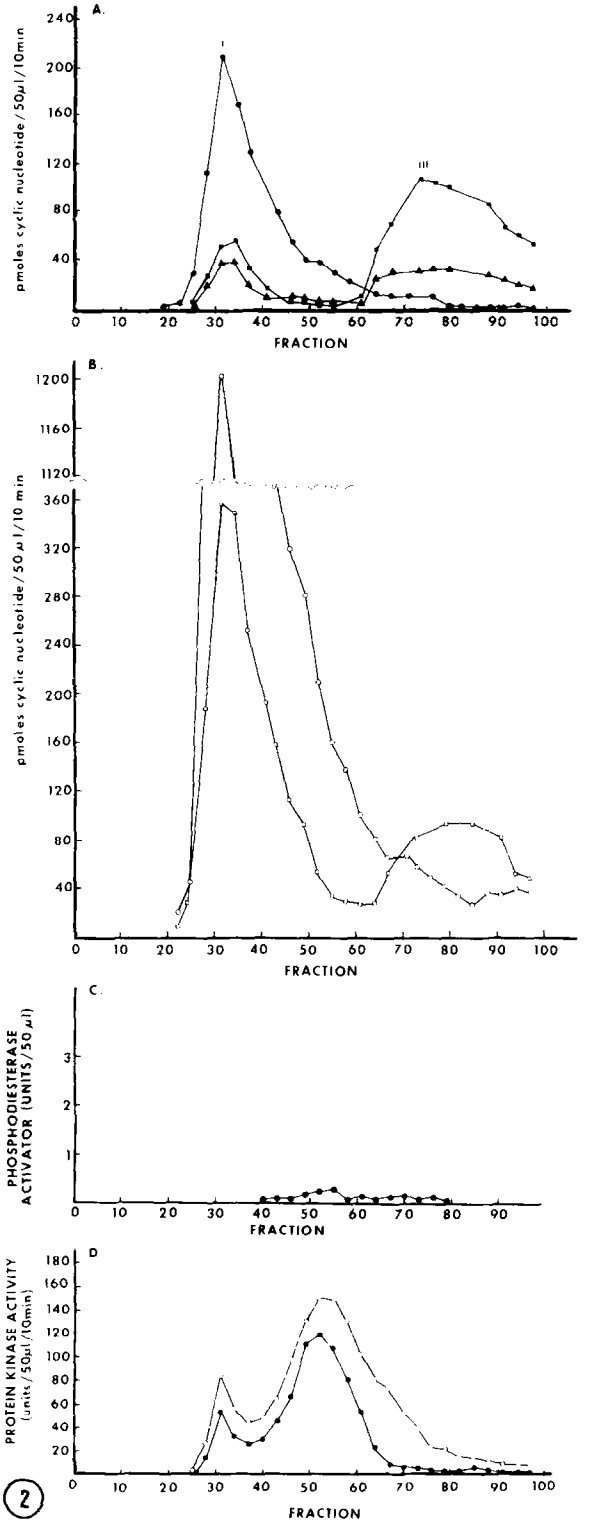
Crude bovine heart phosphodiesterase was prepared by homogenizing 75g of tissue in 5 volumes of buffer containing 10 mM potassium phosphate, pH 7.4 and 4 mM 2-mercaptoethanol. All procedures were done at 4°C. The homogenate was centrifuged at 50,000 x g for 60 min. The crude phosphodiesterase was precipitated with solid ammonium sulfate (40g/100 ml) and centrifuged at 50,000 x g for 15 min. The precipitate was dissolved in a small volume of homogenization buffer and dialyzed overnight against 10 liters of the homogenization buffer. The dialyzed sample was then applied to a column (2 x 25 xm) of DEAE-cellulose which had been equilibrated with the homogenization buffer. The loaded column was washed with 20 mM potassium phosphate buffer, pH 7.4 and 4 mM 2-mercaptoethanol. The column was eluted with a 1 liter gradient of 20-400 mM potassium phosphate, pH 7.4 containing 4 mM 2-mercaptoethanol. Approximately 10 ml fractions were collected and 50 µl aliquots were removed for assay in the standard procedure.

Cyclic AMP-dependent protein kinase was assayed according to the method of Kuo and Greengard (11). The standard assay contained in a final volume of 0.2 ml, 50 mM sodium acetate buffer, (pH 6.0), 40 µg of arginine-rich histone (Worthington, type HA), 5 µM [γ-^{32}P]ATP (10^6 counts per min), 10 mM magnesium acetate, and an aliquot of the eluted fractions containing the cyclic AMP-dependent protein kinase in the absence or presence of 5 µM cyclic AMP. Incubations were carried out at 30° for 10 min and the reaction was terminated, and the degree of histone phosphorylation was measured as described earlier (11). One unit of protein kinase activity is that amount of protein kinase which will transfer one pmole of $^{32}P_i$ to the histone during the incubation.

Phosphodiesterase activator was assayed in the eluted fractions by heating 2 ml aliquots of the fractions in a boiling water bath for 10 min. Following cooling, precipitates were removed by centrifugation at 1200 x g for 15 min. The supernatants were dialyzed extensively against 20 mM potassium phosphate, pH 7.4 and 4 mM 2-mercaptoethanol. One unit of phosphodiesterase activator in the supernatant was defined as that amount which will stimulate the hydrolysis of 10^{-6}M cyclic GMP 50% by the activator-dependent phosphodiesterase (fractions 29 to 35, fig. 1A) in the presence of 20 µM $CaCl_2$ in the standard assay.

RESULTS AND DISCUSSION: The pattern of elution of the bovine heart cyclic nucleotide phosphodiesterases from a column of DEAE-cellulose is shown in Fig. 1A. Peak I, as reported previously (2), is the activator-dependent cyclic nucleotide phosphodiesterase which hydrolyzes approximately 3-fold more cyclic GMP than cyclic AMP. Peak II enzyme is similar to peak I enzyme as it hydrolyzes about 2.5-fold more cyclic GMP than cyclic AMP. The addition of 10^{-6}M cyclic


A
pmoles cyclic nucleotide/50µl/10min
FRACTION
B.
pmoles cyclic nucleotide/50 µl/10 min
FRACTION
C.
PHOSPHODIESTERASE ACTIVATOR (units 50µl)
FRACTION
D
PROTEIN KINASE ACTIVITY (units/50µl/10min)
FRACTION
1


A.
pmoles cyclic nucleotide/50µl/10min
FRACTION
B.
pmoles cyclic nucleotide/50 µl/10 min
FRACTION
C.
PHOSPHODIESTERASE ACTIVATOR (UNITS/50 µl)
FRACTION
D
PROTEIN KINASE ACTIVITY (units/50µl/10min)
FRACTION
2

GMP stimulated the hydrolysis of 10^{-6}M cyclic AMP 160% by the peak II enzyme but inhibited the hydrolysis by peaks I and III (Fig. 1A). Peak III is a relatively cyclic AMP-specific enzyme.

The addition of a saturating amount of phosphodiesterase activator and Ca^{2+} increased the activity of peak I enzyme 6-7 fold (Fig. 1B). The profile suggests that cyclic GMP phosphodiesterase activity is only associated with the peak I enzyme, however a portion of the activity eluted throughout all remaining fractions. Similarly cyclic AMP hydrolysis associated with peak I enzyme was increased by activator in all fractions prior to the peak III enzyme. No distinct peak II enzyme was observed.

Phosphodiesterase activator co-purified with the peak II enzyme (Fig. 1C). As the activity of phosphodiesterase activator is dependent on Ca^{2+} (6,7), a

Fig. 1A. DEAE-cellulose chromatography of cyclic nucleotide phosphodiesterase obtained from bovine heart. Aliquots (50 µl) of the indicated fractions were assayed in the standard procedure with 10^{-6}M cyclic [^{3}H] GMP in the absence (-●-) or presence of 100 µM EGTA, pH 7.4 (--○--). The fractions were also assayed with 10^{-6}M cyclic [^{3}H] AMP alone (-■-) or with 100 µM EGTA, pH 7.4 (--□--) or with 10^{-6}M cyclic GMP (-▲-).

B. Effects of the addition of saturating amounts of phosphodiesterase activator (10 µg) and $CaCl_2$ (100 µM) on phosphodiesterase activity in the fractions from A. Aliquots (50 µl) of the fractions were assayed with activator and $CaCl_2$ with 10^{-6}M cyclic [^{3}H] GMP (-○-) or 10^{-6}M cyclic [^{3}H] AMP (-□-) as substrates in the standard procedure.

C. Phosphodiesterase activator present in the fractions from A. The fractions were assayed as indicated in Methods.

D. Cyclic AMP-dependent protein kinase present in the fractions from A. Aliquots (50 µl) of the indicated fractions were assayed as in Methods in the absence (-●-) or presence of 5 µM cyclic AMP (-○-).

Fig. 2A. DEAE-cellulose chromatography of cyclic nucleotide phosphodiesterase obtained from bovine heart. The animal was kept in an open feed lot exposed continually to freezing night temperatures (-5 to 0°C) for approximately 10 days prior to slaughter. Aliquots (50 µl) of the indicated fractions were assayed with 10^{-6}M cyclic [^{3}H] GMP (-●-) or with 10^{-6}M cyclic [^{3}H] AMP in the absence (-■-) or presence of 10^{-6}M cyclic GMP (-▲-) in the standard procedure.

B. Effects of the addition of saturating amounts of phosphodiesterase activator (10 µg) and $CaCl_2$ (100 µM) on phosphodiesterase activity in the fractions from 2A with the procedure detailed in the legend to Fig. 1B.

C. Phosphodiesterase activator present in fractions from A. The fractions were assayed as indicated in Methods.

D. Cyclic AMP-dependent protein kinase present in fractions from A. Aliquots (50 µl) of the fractions were assayed as indicated in Methods in the absence (-●-) or presence of 5 µM cyclic AMP (-○-).

decrease in enzyme activity following the addition of EGTA has been used as an indication of the dependence of enzyme activity on phosphodiesterase activator. On the addition of 100 μM EGTA (pH 7.4), the activity of peak II decreased approximately 65% while the activities of peaks I and II were unchanged (Fig. 1A). Therefore the peak II enzyme activity apparently represents a mixture of the activator-dependent phosphodiesterase and phosphodiesterase activator.

Cyclic AMP bound to cyclic AMP-dependent protein kinase is resistant to phosphodiesterase activity (12,13), therefore the fractions were assayed for cyclic AMP-dependent protein kinase as its presence might significantly alter the hydrolysis of cyclic AMP. Using the nomenclature of Reimann et al. (14), type I cyclic AMP-dependent protein kinase eluted predominantly with peak I phosphodiesterase while type II cyclic AMP-dependent protein kinase eluted with peak II phosphodiesterase (Fig. 1D).

A seasonal variation in cyclic nucleotide phosphodiesterase activity from bovine heart was observed in cattle kept in open feed lots prior to the time of slaughter. During the summer the elution profile shown in Fig. 1A was routinely observed. However the elution profile shown in Fig. 2A was observed in several animals 10-14 days following the onset of continually freezing night temperatures (-5 to 0°C). Peaks I and III eluted as observed in Fig. 1A however peak II activity was not present. The addition of 10^{-6}M cyclic GMP did not significantly stimulate the hydrolysis of cyclic AMP by any fraction (Fig. 2A) and none of the eluted fractions contained significant amounts of phosphodiesterase activator (Fig. 2C). The addition of a saturating amount of phosphodiesterase activator and Ca^{2+} increased the activity of peak I enzyme 6-7 fold (Fig. 2B), with a profile very similar to that observed in Fig. 1B. The amount of phosphodiesterase activator (Fig. 1C) normally co-purifying with peak II in fractions 49 to 63 (Fig. 1A) was calculated to be sufficient to stimulate fractions 49 to 63 of peak I (Fig. 2A) to produce a peak of enzyme activity of the same approximate magnitude, shape and substrate specificity as was observed in peak II of Fig. 1A. In the presence of saturating or subsaturating levels of phosphodi-

esterase activator (data not shown), the addition of 10^{-6}M cyclic GMP did not stimulate the hydrolysis of 10^{-6}M cyclic AMP by fractions 49 to 63 from Fig. 2A suggesting that some other factor may regulate this stimulation. Type II cyclic AMP-dependent protein kinase eluted in the same position as observed in Fig. 1D, however the activity of the enzyme was less dependent on added cyclic AMP (Fig. 2D).

Cyclic GMP has been shown to displace cyclic AMP bound to the regulatory subunit of cyclic AMP-dependent protein kinases from rat skeletal muscle (15) and human tonsillar lymphocytes (16). As cyclic AMP bound to cyclic AMP-dependent protein kinase is not degraded by phosphodiesterase, cyclic GMP might increase cyclic AMP hydrolysis as shown in Fig. 1A by decreasing the binding of cyclic (^{3}H)AMP to the type II cyclic AMP-dependent protein kinase. The lack of stimulation of cyclic (^{3}H) AMP hydrolysis by added cyclic GMP observed in Fig. 2A might be due in part to alterations in the total binding of cyclic (^{3}H) AMP to the type II cyclic AMP-dependent protein kinase (Fig. 2D) as the enzyme was much less dependent on added cyclic AMP for maximal activity.

Gnegy et al. (17) demonstrated that activator in rat brain is normally approximately equally distributed between the soluble and particulate fractions. Short-term stimulation by catecholamines increased the content of activator in the soluble fraction with a loss from the particulate fraction (18), while chronic blockage of dopamine receptors in rat striata with haloperidol resulted in a marked increase in the level of activator present in the particulate fraction with little change in the soluble fraction (19). Prolonged exposure of cattle to freezing temperatures may lead to prolonged adrenergic stimulation possibly resulting in a decreased level of the activator in the soluble fraction or in association with the activator-dependent phosphodiesterase.

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