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ACTIVATION OF MAMMALIAN CYCLIC AMP PHOSPHODIESTERASES BY TRYPSIN

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Summary

BHK fibroblasts contain two forms of cyclic AMP phosphodiesterase (3′:5′-cyclic nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) as analyzed by linear sucrose gradient fractionation; a 3.6-S form (peak I) and a 6.7-S form (peak II). Peak I is specific for cyclic AMP as substrate and displays Michaelis-Menten kinetics with an apparent K_m of 2—3 μM. Peak II hydrolyzes cyclic GMP and displays anomalous kinetics for cyclic AMP hydrolysis. The activity of isolated peak II for cyclic AMP is increased by storage at 4°C, treatment with trypsin, or treatment with rat brain and BHK fibroblast activator proteins. The activity of isolated peak I is unaffected by these conditions. Linear sucrose gradient fractionation demonstrates that activation of peak II by trypsin leads to the formation of a 3.6-S cyclic AMP-specific enzyme form, possibly peak I.

In contrast to BHK fibroblasts (and most other mammalian tissues), rat uterus contains only one form of cyclic nucleotide phosphodiesterase on linear sucrose gradients, a 7-S form capable of hydrolyzing both cyclic AMP and cyclic GMP. Treatment of rat uterine supernatant with trypsin leads to the appearance of a 4-S, cyclic AMP-specific form with properties similar to that of BHK peak I. These data suggest that the kinetically complex, higher molecular weight cyclic nucleotide phosphodiesterases may consist of more than one catalytically active site and that multiple forms of the enzyme arise through dissociative mechanisms, possibly as a means of in vivo regulation.

Introduction

In most mammalian tissues, cyclic nucleotide phosphodiesterase(s) (3′:5′-cyclic nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) are thought to exist as

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a complex of enzyme forms. The isolation of multiple forms of this enzyme has been accomplished by using a variety of physical separation techniques, and these forms have been shown to differ in their substrate specificity and kinetic properties [1]. The functional significance of multiple forms of this enzyme activity and the relationship between them is not well understood. Certain biological conditions lead to alterations in the activity of one or another of the enzyme forms. For example, incubation of chicken embryonic fibroblasts [2], cultured hepatoma cells [3], or isolated human peripheral blood lymphocytes [4] with 1-methyl-3-isobutylxanthine leads to activation of a specific enzyme form, as does incubation of cloned astrocytoma cells with norepinephrine [5]. Similarly, the postnatal increase in phosphodiesterase activity in rat cerebrum results primarily from an increase in a specific enzyme form [6]. Also, insulin causes activation of a single enzyme form in BHK fibroblasts [7], and activates particulate but not soluble enzyme forms in liver and adipose tissue [8,9]. Since neither the origin nor the function of the multiple forms of the enzyme is known, agents which activate specific forms and studies of their mechanisms could be useful in solving these problems.

Activation of cyclic AMP phosphodiesterase by trypsin has been reported for enzyme from brain [10—12] and from rod outer segment [13]. In addition, trypsin alters the cyclic nucleotide content of lymphocytes [14], the activity of a membrane-bound phosphodiesterase in fibroblasts [15], and promotes fibroblast [16] and lymphocyte [17] cell growth. In this paper we have studied the activation by trypsin of a higher molecular weight enzyme from BHK fibroblasts, which contain multiple enzyme forms, and from rat uteri, which contain only a single enzyme form. The results of these activations and the production of similar lower molecular weight enzymes are discussed in relation to the biochemical nature of cyclic nucleotide phosphodiesterases. Preliminary reports of this work have been published previously [18,19].

Materials and Methods

Materials

[^{3}H]Adenosine 3′,5′-cyclic monophosphate (cyclic [8-^{3}H]AMP, 20 Ci/mmol) was purchased from Schwarz-Mann, Orangeburg, N.Y. [^{3}H]guanosine 3′:5′-cyclic monophosphate (cyclic [G-^{3}H]GMP, 8.4 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. Cyclic [^{3}H]AMP and cyclic [^{3}H]GMP were purified by Dowex I-X8 (200—400 mesh) anion-exchange chromatography and stored at −20°C in acidic 50% ethanol prior to use. Snake (*Ophiophagus hannah*) venom, cyclic AMP, and cyclic GMP were from Sigma, St. Louis, Mo. Trypsin (TRTPCK, chymotrypsin-free, 266 units/mg) was from Worthington Biochemical, Freehold, N.J. Lima bean trypsin inhibitor was from P-L Biochemicals, Milwaukee, Wisc. Dowex I-X8 (200—400 mesh) was from Biorad Laboratories, Richmond, Calif. Minimal essential medium was from Grand Island Biological, Grand Island, N.Y. All other chemicals were of commercial analytical grade quality.

Methods

Preparation of cyclic nucleotide phosphodiesterases from BHK fibroblasts.

Baby hamster kidney fibroblasts (BHK 21/c13) were obtained from Flow Laboratories, Inc. The cells were grown in minimal essential medium containing 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) buffer supplemented with 10% fetal calf serum. Surface cultures of BHK cells were grown to a high density ($>30 \cdot 10^6$ cells/150 cm^2 flask) and removed from the substrate with a rubber scraper. The cells were pelleted by low speed centrifugation, resuspended in 50 mM Tris-HCl (pH 7.4) buffer and sonically disrupted for 30 s at setting No. 50 (80 W) in a Biosonik IV (VWR Scientific) sonicator. The resulting crude suspension was centrifuged at 105 000 $\times g$ for 60 min and the supernatant fraction (which contained greater than 95% of the high affinity cyclic AMP and cyclic GMP activity) was used as the source of phosphodiesterase activity. High affinity cyclic nucleotide phosphodiesterase is defined as the hydrolytic activity measured at 0.25 μM substrate concentration.

Preparation of cyclic nucleotide phosphodiesterases from rat uteri. Female Sprague-Dawley Rats (20-22 days old) were obtained from Texas Inbred Mouse Co., Houston, Texas. Rats were killed by decapitation; the uteri were removed, stripped of adhering fat and mesentery and homogenized in 40 mM Tris-HCl (pH 8.0) with a Duall type ground glass homogenizer as one uterus per ml. Supernatant preparations containing greater than 95% of cyclic AMP and cyclic GMP activity were then prepared by centrifugation at 105 000 $\times g$ for 60 min.

Assay of cyclic nucleotide phosphodiesterase activity. A modified procedure [20] of the radioisotopic method of Thompson and Applemen [21] was employed for the assay of cyclic GMP phosphodiesterase activity and for the assay of low K_m (0.25 μM) cyclic AMP phosphodiesterase activity. Reaction mixtures (0.4 ml) contained 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.25 μM cyclic [^{3}H]AMP or 1 μM cyclic [^{3}H]GMP, and enzyme preparation as indicated in the figure legends. Reactions were incubated at 30°C for 15 min and terminated by boiling for 1 min. 0.1 ml (0.5 mg/ml) snake venom (*Ophiophagus hannah*) was added to the chilled samples and incubated for 10 min at 30°C. 1 ml of methanol was added to each sample and ^{3}H-labeled nucleosides were separated from unreacted cyclic nucleotides by chromatography over a (5 X 14 mm) column of Dowex I-X8 resin as described elsewhere [20]. Each column was eluted directly into a scintillation vial and rinsed with 1 ml of methanol. The entire eluate and wash were counted in 8 ml Aquasol by liquid scintillation techniques. Using this procedure, greater than 90% of the reaction product was recovered in all cases. High K_m cyclic AMP phosphodiesterase activity was assayed at 200 μM cyclic AMP substrate concentration by the firefly luciferase method of Weiss et al. [22].

Preparation of brain activator protein. 23 rat brains (29 g tissue) were homogenized in cold distilled water in a 1 : 3 (w/v) ratio using a polytron homogenizer. The pH was adjusted to 5.9 with acetic acid and the homogenate was centrigued at 13 000 $\times g$ for 30 min. The supernatant solution was subjected to 0—50% and 50—85% fractionation at pH 4.1. The 50—85% $(NH_4)_2SO_4$ pellet was resuspended in 5 ml 10 mM glycyl-glycine and neutralized with 2 M Tris-HCl (pH 8.0). The solution was heated at 80°C for 3 min with stirring, the precipitated proteins removed by centrifugation at 13 000 $\times g$ for 30 min, and the heat treatment and centrifugation repeated. The final supernatant fraction was brought to 30% with ethylene glycol and stored at —20°C.

Assay of phosphodiesterase activator activity. Activation of phosphodiesterase by rat brain activator was determined by the method of Weiss et al. [22] with the inclusion of 10 μM $CaCl_2$ and an amount of activator protein as indicated. Control samples contained an equivalent amount of bovine serum albumin.

Sucrose gradient density centrifugation. 5 ml linear 5—20% sucrose gradients in 50 mM Tris-HCl buffer (pH 7.4) were prepared in polyallomer centrifuge tubes. The enzyme preparations (0.2 ml) were layered on the gradients and centrifuged at 192 000 $\times g$ (r maximal) in a Beckman SW 50.1 rotor for 16 h at 4°C. The gradients were fractionated into 0.2-ml aliquots using an Isco Model 640 fractionator. γ-[^{14}C]Globulin (7 S) and [^{14}C]ovalbumin (3.6 S) were used as sedimentation profile markers.

Protein determinations. Proteins were measured by the method of Schacterle and Pollack [23] using bovine serum albumin as standard.

Results

Activation of a specific form of BHK cyclic AMP phosphodiesterase

Greater than 95% of the cyclic AMP phosphodiesterase activity of BHK cells (0.25 μM or 200 μM substrate) is in the soluble portion of the cell. Although activity varies with the growth stage of the cells, it remains soluble throughout the cycle [7]. Sucrose gradient fractionation of a 100 000 $\times g$ supernatant of high density BHK cells displays two peaks of cyclic AMP phosphodiesterase activity: a 3.6-S form and a 6.7-S form, and one form of cyclic GMP phosphodiesterase activity which sediments at 7 S (see control, Fig. 3). The same enzyme profile was obtained when cells were sonically disrupted in and sedimented through buffer containing the protease inhibitors, 0.6 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, and 0.01% lima bean trypsin inhibitor.

The activity of cyclic AMP phosphodiesterase in BHK homogenates increases 2-fold after 24—48 h storage at 4°C in a manner previously shown for homogenates of human peripheral blood lymphocytes [24] and rat liver homogenates [25]. The increased activity in human peripheral blood lymphocytes upon storage results in a large increase in the activity of a specific form of the enzyme isolated by sucrose gradient fractionation [24]. The possibility that increased enzyme activity in BHK cells might also result from a change in the activity of a specific enzyme form was investigated. The 3.6-S (peak I) and the 6.7-S (peak II) enzyme forms were isolated from BHK cells by linear sucrose gradient fractionation and the activity of the isolated enzyme forms was measured on the day of isolation and for 3 days thereafter during storage at 4°C. The results (Fig. 1) show that only peak II, the heavy molecular weight enzyme form, increased in activity during storage; whereas, the activity of peak I was relatively unaffected. Unlike liver homogenates [25], the increase in activity was not prevented by dialysis.

Since proteolysis could be involved in storage activation, and since trypsin is known to activate enzyme in crude brain preparations [10,11], trypsin was tested for its effects on specific enzyme forms in BHK cells. The two enzyme forms were fractionated on a linear sucrose gradient and each fraction assayed

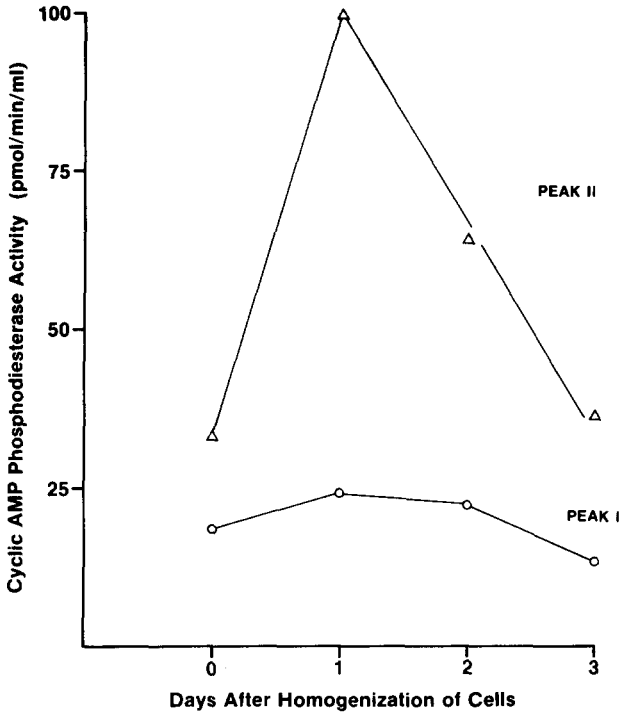

Fig. 1. Effect of 4°C storage on activity of BHK cyclic AMP phosphodiesterase forms separated by linear sucrose gradient fractionation. The 105 000 × *g* supernatant from 12 · 10^6 BHK cells was fractionated by linear sucrose gradient centrifugation and assayed for cyclic AMP phosphodiesterase activity at 0.25 μM cyclic AMP. Two peaks of enzyme activity were observed; one at 3.6-S (peak I) and another at 6.5 S (peak II). The peak fractions were stored at 4°C and assayed for cyclic AMP phosphodiesterase activity daily for the next 3 days. The activities of the isolated 3.6-S fraction (peak I) and the activities of the isolated 6.5-S fraction (peak II) are presented.

in the presence and absence of trypsin (1.25 μg/ml). The results (Fig. 2A) show that in BHK cells, only peak II is activated by trypsin, whereas peak I is unaffected. Masked, or inactive phosphodiesterase activity was not uncovered elsewhere in the gradient by assaying in the presence of trypsin.

A Ca^{2+}-dependent protein activator of phosphodiesterase was previously shown to differentially activate multiple forms of the brain enzyme separated by isoelectric focusing [26]. In BHK cells, this protein activator, isolated from brain, activates only the peak II enzyme form and has no effect on the activity of peak I (Fig. 2B). Identical results were obtained with the protein activator isolated from BHK cells.

Effect of activation on the enzyme sedimentation profile

BHK supernatant fluid treated with trypsin was fractionated by linear sucrose gradient sedimentation and compared to an untreated preparation. The results (Fig. 3) show that trypsin activation of cyclic AMP phosphodiesterase activity in BHK supernatant fluid led to a change in the sedimentation profile of the enzyme. There was a decrease in enzyme activity sedimenting at 6.7 S and an increase in activity sedimenting at 3.6 S. Under these conditions, in

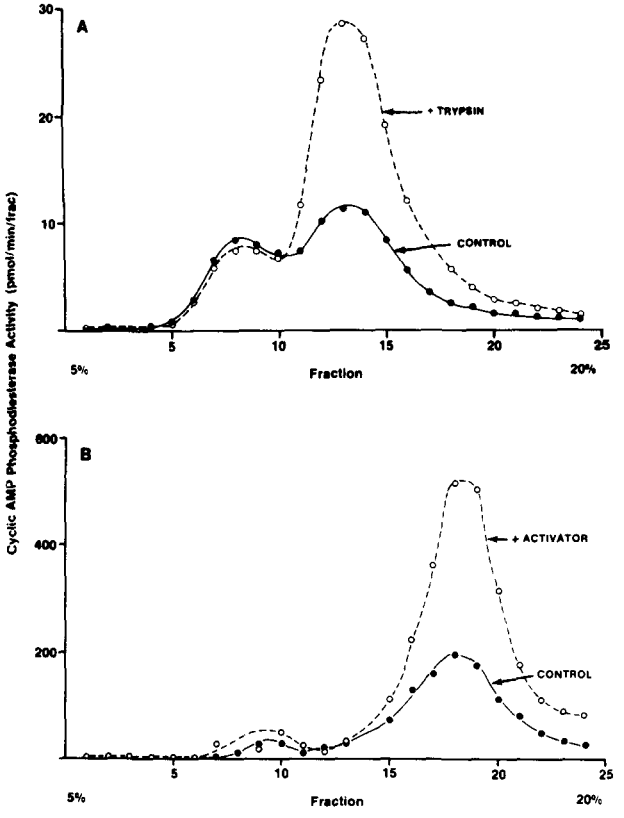

Fig. 2. (A) Effect of trypsin and brain activator on BHK cyclic AMP phosphodiesterase fractionated on a linear sucrose gradient. (A) Effect of trypsin. The 105 000 × *g* supernatant from $30 \cdot 10^6$ BHK cells (6 mg protein) was sedimented through a linear 5—20% sucrose gradient and fractionated into 0.2-ml fractions. A 20 μl aliquot of each fraction was assayed for cyclic AMP phosphodiesterase activity at 0.25 μM cyclic AMP by the radioisotope procedure either in the absence (●) or presence (○) of 500 ng trypsin. (B) Effect of brain activator. Enzyme supernatant from $3.8 \cdot 10^6$ BHK cells (1.1 mg of protein) was fractionated through sucrose into 0.2-ml fractions. A 50 μl aliquot of each fraction was assayed for cyclic AMP phosphodiesterase activity at 200 μM cyclic AMP by the firefly luciferase method in the absence (●) or presence (○) of 14 ng rat brain activator protein. Control samples contained an equivalent amount of bovine serum albumin in the assay.

which total cyclic AMP phosphodiesterase activity was enhanced 31% by trypsin, total cyclic GMP phosphodiesterase activity was not activated by trypsin, but instead, was decreased by 42%. Cyclic GMP phosphodiesterase still sedimented as a single peak of activity following trypsin treatment of the supernatant, but is appeared to be slightly smaller, since its sedimentation value shifted from 7 to 6.7 S (Fig. 3C). The increase in activity, sedimenting at 3.6 S following trypsin treatment of the supernatant is apparent over a wide range of protein concentrations applied to the gradient (0.6—6 mg) and this increase is evident when the data is expressed not only as total activity, but as specific activity as well (Fig. 3, and unpublished data).

When BHK supernatant fluid was treated with brain activator and fractionated on sucrose gradients, an increase in cyclic AMP phosphodiesterase

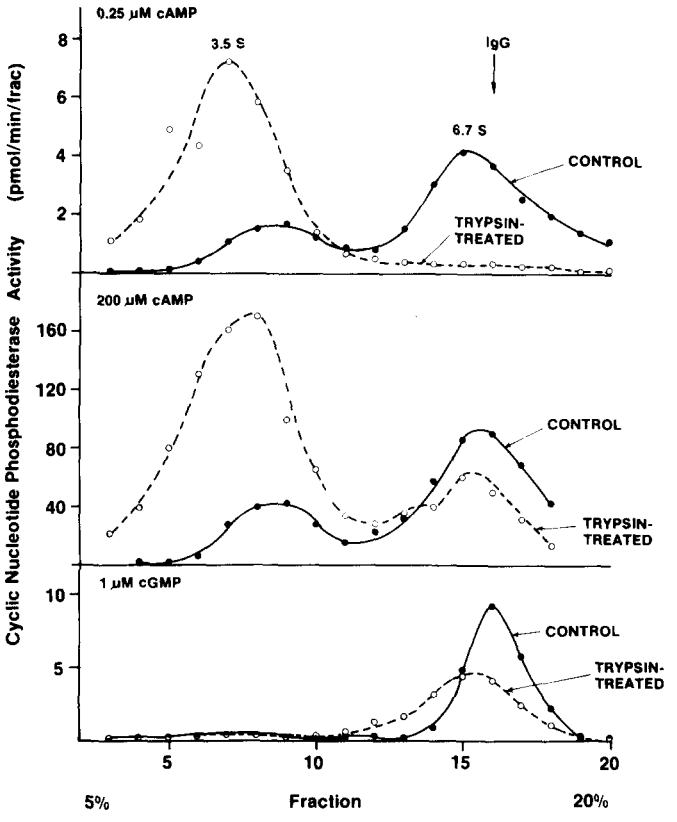

Fig. 3. Effect of trypsin treatment of BHK supernatant on sedimentation profile of cyclic nucleotide phosphodiesterases. Enzyme supernatant was prepared from $36 \cdot 10^6$ BHK cells sonicated in 0.7 ml buffer and the supernatant was then desalted on a Sephadex G-25 column (Pharmacia PD-10), and collected in a total volume of 1 ml. A 250 μl aliquot of the desalted supernatant (2.5 mg protein) was incubated for 2 min at 30°C with 4 μg (16 μg/ml) trypsin and the reaction was stopped by the addition of 20 μg lima bean trypsin inhibitor. As a control, 250 μl desalted supernatant was also incubated for 2 min at 30°C, and this control tube also received 20 μg lima bean trypsin inhibitor. The treated supernatants were applied to sucrose gradients and fractionated. Each fraction was assayed for cyclic nucleotide phosphodiesterase activity at 0.25 μM cyclic AMP (top panel), 200 μM cyclic AMP (cAMP) (middle panel), and 1 μM cyclic GMP, (bottom panel).

enzyme activity was seen at 6.7 S, but there was no change in activity sedimenting at 3.6 S.

Effect of activation mechanisms on physical sedimentation properties of isolated enzyme forms

The results of BHK supernatant fluid suggest that trypsin activation may result from conversion of the peak II enzyme form to the peak I form, or to a form with sedimentation characteristics identical to peak I. The possibility that activation may result from a physical conversion mechanism was further investigated by isolating the two forms of cyclic AMP phosphodiesterase by sucrose gradient fractionation, and resedimenting them following storage at 4°C, treatment with trypsin, or treatment with brain activator.

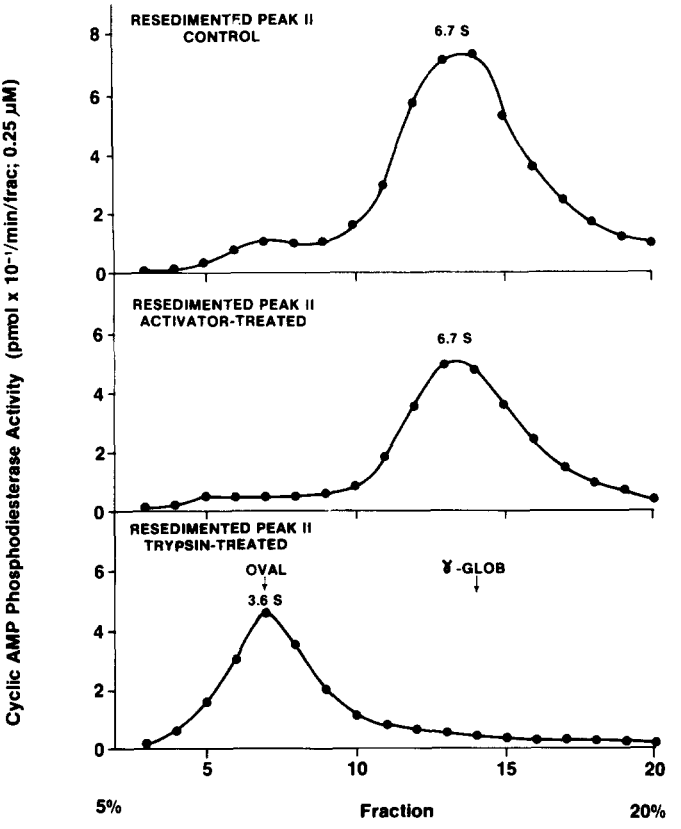

Fig. 4. Resedimentation of isolated BHK peak II following treatmetns with brain activator and trypsin. Enzyme supernatant prepared from $31 \cdot 10^6$ BHK cells was fractionated on a sucrose gradient. The fractions were assayed for cyclic AMP phosphodiesterase activity at 0.25 μM cyclic AMP and fractions surrounding the two peaks of activity (3.6 and 6.7 S) were pooled separately, desalted on a Sephadex G-25 column (Pharmacia PD-10), and brought to 10 μM $CaCl_2$. Aliquots (0.25 ml) of the pooled, desalted peaks were treated as follows: 1, control, no treatment; 2, activator-treated, 60 min incubation at 0°C with 3.6 μg of brain activator; and 3, trypsin-treated, 2 min incubation at 30°C with 3 μg (12 μg/ml) trypsin, followed by the addition of 15 μg lima bean trypsin inhibitor. 0.2 ml of each treated aliquot was then sedimented and fractionated on new sucrose gradients, and the fractions were assayed for cyclic AMP phosphodiesterase activity at 0.25 μM cyclic AMP. Results show the resedimented control peak II, (top panel) resedimented activator-treated peak II (middle panel), resedimented trypsin-treated peak II (bottom panel). The resedimented peak I fractions are not shown.

Isolated peak II treated with brain activator resediments as a 6.7-S form, whereas after treatment with trypsin, it resediments as a 3.6-S form (Fig. 4). The sedimentation properties of isolated peak I were unaffected by treatment with trypsin or activator (unpublished data). When isolated peaks I and II are stored at 4°C for 3 days and then analyzed by sucrose gradient sedimentation, peak I resediments only as a 3.6-S form, whereas peak II showed peaks of activity at 3.6 and 6.7 S.

Mechanism of activation by trypsin

In brain, trypsin has been reported to activate phosphodiesterase through both an increase in the V and a decrease in the apparent K_m of the enzyme

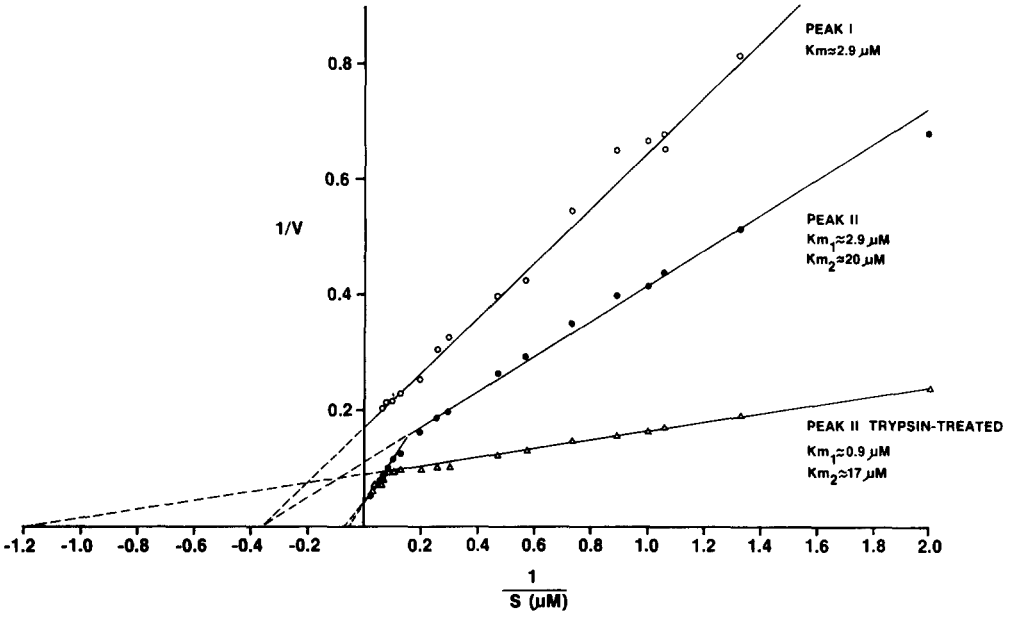

Fig. 5. Representative Lineweaver-Burk plots of separated BHK cyclic AMP phosphodiesterase forms and the effect of trypsin on isolated peak II. Peak fractions from the fractionated sucrose gradient presented in Fig. 2A, control, were pooled (peak I, fractions 7—8; peak II, fractions 12—15). Pooled peak II was diluted to 3.6 ml with 40 mM Tris-HCl (pH 8.0) buffer, and split into two equal portions. Each portion was incubated for 1 min at 30°C either without (control) or with the addition of 7.5 μg (4.2 μg/ml) of trypsin (trypsin-treated). At the end of the 1 min incubation, 100 μg lima bean trypsin inhibitor was added to both the control and trypsin-treated tubes. Enzyme activity was assayed by the radioisotope procedure, at substrate concentrations from 0.5 to 50 μM. Apparent Michaelis constants (K_m) and maximum velocities (V) were determined from the linear portions of the plots by linear regression analysis.

[10,11]. We investigated the effects of trypsin activation on the kinetics of cyclic AMP phosphodiesterase in BHK cells. Kinetic analysis of isolated BHK cyclic AMP phosphodiesterase forms is shown in Fig. 5. Peak I displays Michaelis-Menten kinetics with an apparent K_m of 2—3 μM; whereas peak II displays anomalous kinetics with extrapolated apparent K_m values of 2—3 and 20 μM. Treatment of isolated peak II with trypsin leads to a 3-fold decrease in the low K_m of the system, with little or no increase in the V (Fig. 5).

Brain activator and trypsin appear not to be synergistic in their ability to activate phosphodiesterase. When BHK supernatant is treated with trypsin, fractionated by sucrose gradient sedimentation, and assayed for phosphodiesterase activity in the presence and absence of brain activator, peak II was no longer susceptible to activation by brain activator.

Effect of trypsin on sedimentation profile of uterine cyclic nucleotide phosphodiesterase

Rat uterus contains 95% of the total cyclic nucleotide phosphodiesterase activity in the soluble form [27]. Analysis of the soluble enzyme activity by sucrose gradient sedimentation (Fig. 6 and ref. 27), DEAE-cellulose chromatography [27] and agarose gel filtration [28] indicates the presence of only one enzyme form, when assayed with either cyclic AMP or cyclic GMP as substrate.

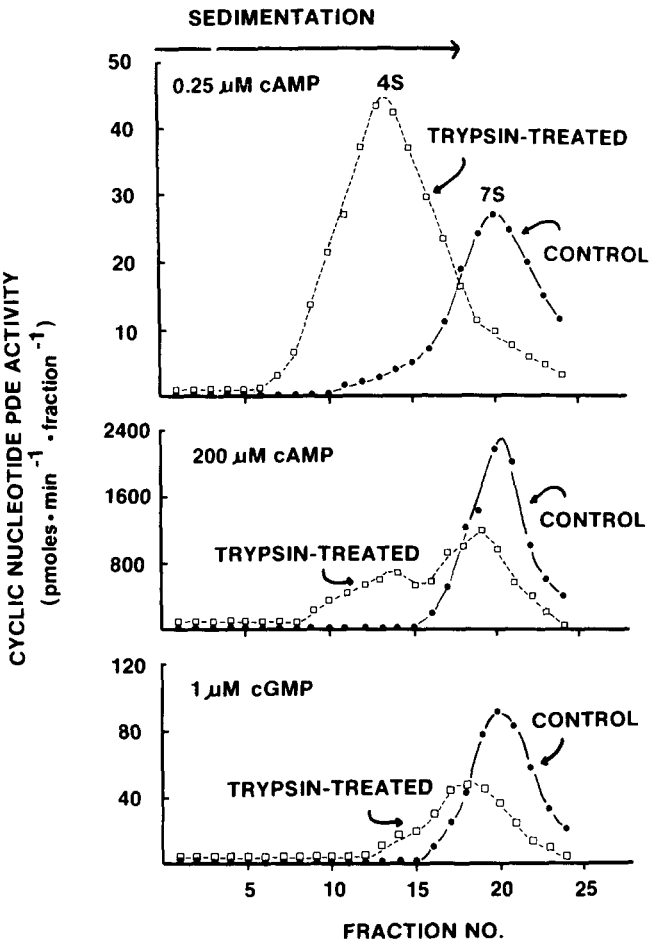

Fig. 6. Effect of trypsin treatment of rat uterine supernatant on sedimentation profile of cyclic nucleotide phosphodiesterase. Rat uterus supernatant protein (600 μg) was incubated either with (trypsin-treated) or without (control) 4 μg (20 μg/ml) trypsin for 2 min at 30°C, followed by the addition of a 5-fold excess of lima bean trypsin inhibitor. Samples were fractionated on sucrose gradients and each fraction was assayed for cyclic nucleotide phosphodiesterase activity at 0.25 μM cyclic AMP (top panel), 200 μM cyclic AMP (cAMP) (middle panel) and 1 μM cyclic GMP (bottom panel).

Rat uterine phosphodiesterase normally sediments at about 7-S in sucrose gradients (Fig. 6); however, limited trypsin treatment of rat uterine supernatant results in the appearance of two phosphodiesterase forms isolated by sucrose gradient sedimentation: a 4-S, low K_m, cyclic AMP-specific form, and a 6.7-S, cyclic AMP-cyclic GMP non-specific form (Fig. 6).

The uterine single enzyme form displays anomalous kinetics for cyclic AMP hydrolysis with extrapolated apparent K_m values of 3 and 20 μM [19]. Kinetic analysis of the two enzyme forms isolated after trypsin treatment, reveals that the 6.7-S form maintained the same anomalous kinetics for cyclic AMP hydrolysis, with extrapolated apparent K_m values of 2 and 17 μM, but the 4-S form displayed linear Michaelis-Menten kinetics with an apparent K_m of 1 μM. Hence, trypsin treatment of rat uterine supernatant produces two enzyme

forms that are similar to the two forms isolated from sonically disrupted BHK fibroblasts, not only in their physical sedimentation characteristics, but in their kinetic properties and substrate affinities as well.

Discussion

Sucrose density gradient centrifugation of the supernatant of sonically disrupted BHK fibroblasts reveals multiple forms of cyclic nucleotide phosphodiesterase activity. Two forms of cyclic AMP phosphodiesterase activity are detected, one sedimenting at 3.6 S and the other at 6.7 S, and one peak of cyclic GMP phosphodiesterase activity is observed with a sedimentation coefficient of 7-S. Cyclic AMP phosphodiesterase activity in BHK fibroblasts can be activated by storage of homogenate at 4°C for one to several days, limited trypsin proteolysis, or addition of a heat-stable protein activator. Of the two forms of cyclic AMP phosphodiesterase, only the peak II (6.7-S) form is susceptible to activation by these procedures; the peak I (3.6-S) form is unaltered under the same conditions. Protease activity has been of some concern by several investigators while analyzing multiple forms of cyclic nucleotide phosphodiesterase [11,29—32]. Activation of phosphodiesterase activity by storage of homogenate at 4°C has been noted previously for the human lymphocyte and rat liver systems [24,25,30]. In human lymphocytes, storage of homogenates is accompanied by the loss of a 5.5-S enzyme form and an increase in activity sedimenting as a 3.6-S form [24]. In this paper we show that in BHK fibroblasts, storage of an isolated 6.7-S form, or treatment of this form with trypsin, also results in its conversion to a lower molecular weight, 3.6-S form. Recently, a serine protease was identified in liver lysosomes, which mimics the actions of trypsin on activation of brain and liver phosphodiesterases (Sakai, T., personal communication). These results taken together suggest that storage activation may result from the action of endogenous proteases.

The mechanism of activation of phosphodiesterases by trypsin is not fully understood. In this paper we show that activation by trypsin is selective; it will only activate the enzyme form which can also be activated by protein activator, and has no effect on that form which can not. These findings confirm those of Sakai et al. [11] for the rat brain system. We also find that phosphodiesterase that has been activated by trypsin is no longer susceptible to activation by protein activator, and this too, confirms results on the rat brain system [11]. In BHK fibroblasts, but not brain [10,11], trypsin activation results primarily in a 3-fold lowering of the apparent K_m, with little effect on the V.

Of the variety of mechanisms which could explain the trypsin-activating effect, the simplest is that cytosolic phosphodiesterase exists as a single enzyme form with multiple catalytic sites and that multiple forms are produced by limited proteolysis. We cannot distinguish this mechanism from that of a single form possessing only one catalytic site and its anomalous kinetic behavior resulting from proteolytic conversion by partial digestion either during cell breakage or during incubation for activity analysis. Recent studies using highly purified enzyme forms from dog kidney [45] suggest this as a possibility since both the near homogeneous forms display linear kinetics with cyclic AMP as substrate (2.8-S form, $K_m \simeq 1$ μM; 6-S form, $K_m \simeq 60$ μM). Hence, the anoma-

lous kinetics observed in crude and partially purified systems could result from incubation artifacts.

Alternatively, trypsin could act in a more indirect manner. It has been proposed by others for brain and liver tissues [33,30] that trypsin may destroy an inhibitor bound to the enzyme. An inhibitory brain protein has recently been described [34]; however, this inhibitor appears not to bind to the enzyme and has no effect on basal activity, but rather binds to the calcium-dependent activator protein and prevents activation. Therefore, it is unlikely that this inhibitor is involved in trypsin activation. However, the existence of other inhibitors which interact directly with the enzyme still remains a possibility. Another mechanism might include tryptic activation of inactive proenzyme which could lead to covalent modification of cyclic nucleotide phosphodiesterases. A proenzyme of protein kinase which is activated by trypsin has recently been described in rat liver [35].

It is of interest that in addition to trypsin a variety of other factors and agents seem to selectively modulate activity of the higher molecular weight, non-substrate specific phosphodiesterases. This list includes the heat-stable protein activator [36] whose effects on the kinetics of the enzyme are poorly understood [10,11,26,37—41] and a series of agents with detergent-like actions including the vitamin E derivative sodium α-tocopherol phosphate [11], phosphatidylinositol, lysophosphatidylcholine, sodium dodecyl sulfate, and several fatty acids [42,43]. Since many of these agents have detergent-like action, it is possible that, as postulated for trypsin, they too may act by changing the conformation of the enzyme to expose an additional catalytic site(s), or by removing material which may be inhibiting the catalytic site(s) of the enzyme.

Activation of cyclic AMP phosphodiesterase by trypsin is always accompanied by a reduction in the enzyme molecular weight [11,13,44]. Our observation that in BHK cells, activation by trypsin produces more low K_m cyclic AMP-specific enzyme activity sedimenting at 3.6-S suggests that the initial low molecular weight, low K_m enzyme form may have been derived from the higher molecular weight enzyme form. This may be true of other tissues that display multiple cyclic nucleotide phosphodiesterase forms as well, as suggested for the rat erythrocyte [29]. Whether the 3.6-S form produced by trypsin treatment is identical in all respects to the 3.6-S form present initially in the tissue homogenates is still a question that needs to be resolved. Kinetic analysis of the system (Fig. 5) would tend to indicate that they may not be identical, since activation by trypsin produces an even lower K_m than the original 3.6-S form, but these data must be interpreted with caution since the activatable enzyme displays non-linear kinetics.

It is clear that the initial appearance of multiple enzyme forms makes these studies difficult to interpret; however, by use of the rat uterus, which possesses a single form of soluble phosphodiesterase activity, we clearly demonstrate that a low K_m, cyclic AMP-specific, low molecular weight enzyme form can be derived from a non-specific, higher molecular weight form upon activation by trypsin. Although this does not prove that multiple forms are so derived in other tissues, we nevertheless conclude from these studies that mammalian cyclic nucleotide phosphodiesterases may normally exist as a single higher molecular weight enzyme species possessing multiple catalytic sites that can be

converted by proteolytic and possibly other mechanisms to low and high molecular weight multiple forms. We speculate that in vivo these conversions are a means of cellular regulation and that in vitro these conversions are superimposed on the actual state of the enzyme at the time of cell disruption. We have no evidence at this time that lower molecular weight enzyme forms can be interconverted to the higher molecular weight system. In an earlier report from this laboratory [46], we demonstrated that in the rat kidney system, either single or multiple enzyme forms could be obtained when different methodological procedures were used to isolate the enzyme(s), and this observation is consistent with our current conclusions.

The role of proteolysis as a biological regulatory mechanism is becoming increasingly apparent [47]. In addition to cyclic nucleotide phosphodiesterases, trypsin and other proteolytic enzymes are known to activate other important cellular regulatory enzymes, such as liver fructose biphosphatase [48], muscle phosphorylase [49], and fibroblast adenylate cyclase [50]. Proteolytic action also appears to be responsible for multiple forms observed for *Escherichia coli* nitrate reductase [51], *Drosophila* DNA polymerase [52], and the rat uterus estrogen receptor [53]. Hence, proteolytic activation of cyclic nucleotide phosphodiesterase may provide an additional mechanism for the cellular regulation of this enzyme system.

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References

1 Appleman, M.M., Thompson, W.J. and Russell, T.R. (1973) Adv. Cyclic Nucl. Res. 3, 65—98
2 Russell, T.R. and Pastan, I.H. (1974) J. Biol. Chem. 24, 7764—7769
3 Ross, P.S., Manganiello, V.C. and Vaughan, M. (1977) J. Biol. Chem. 252, 1448—1452
4 Thompson, W.J., Ross, C.P., Hersh, E.M., Strada, S.J. and Epstein, P.M. (1977) Fed. Proc. 36, 283
5 Uzunov, P., Shein, H.M. and Weiss, B. (1973) Science 180, 304—306
6 Strada, S.J., Uzunov, P. and Weiss, B. (1974) J. Neurochem. 23, 1097—1103
7 Thompson, W.J. and Strada, S.J. (1978) in Receptors and Hormone Action (Birnbaumer, L. and O'Malley, B., eds.), Vol. III, pp. 553—577, Academic Press, New York
8 Thompson, W.J., Little, S.A. and Williams, R.H. (1973) Biochemistry 12, 1889—1894
9 Sakai, T., Thompson, W.J., Lavis, V.R. and Williams, R.H. (1974) Arch. Biochem. Biophys. 162, 331—339
10 Cheung, W.Y. (1971) J. Biol. Chem. 246, 2859—2869
11 Sakai, T., Yamanaka, H., Tanaka, R., Makino, H. and Kasai, H. (1977) Biochim. Biophys. Acta 483, 121—134
12 Hintze, R., Martin-Esteve, J. and Calvet, F. (1976) An. Quim. 72, 1026
13 Miki, N., Baraban, J.M., Keirns, J.J., Boyce, J.J. and Bitensky, M.W. (1975) J. Biol. Chem. 250, 6320—6327
14 Schneyour, A., Patt, Y. and Trainen, N. (1976) J. Immunol. 117, 2143—2149
15 Russell, T. and Pastan, I. (1973) J. Biol. Chem. 248, 5835—5840
16 Sefton, B. and Rubin, H. (1971) Proc. Natl. Acad. Sci. U.S. 68, 3154—3157
17 Kaplan, J.G. and Bona, C. (1974) Exp. Cell Res. 88, 388—394

18 Epstein, P.M., Pledger, W.J., Thompson, W.J. and Strada, S.J. (1977) Fed. Proc. 36, 283
19 Strada, S.J., Gardner, E.A., Epstein, P.M., Thompson, W.J. and Stancel, G.M. (1977) The Pharmacologist 19, 203
20 Epstein, P.M., Mills, J.S., Ross, C.P., Strada, S.J., Hersh, E.M. and Thompson, W.J. (1977) Cancer Res. 37, 4016—4021
21 Thompson, W.J. and Appleman, M.M. (1971) Biochemistry 10, 311—316
22 Weiss, B., Lehne, R. and Strada, S.J. (1972) Anal. Biochem. 45, 222—235
23 Schacterle, G.R. and Pollack, R.L. (1973) Anal. Biochem. 51, 654—655
24 Thompson, W.J., Ross, C.P., Pledger, W.J., Strada, S.J., Banner, R.L. and Hersh, E.M. (1976) J. Biol. Chem. 251, 4922—4929
25 Thompson, W.J. and Williams, R.H. (1974) Arch. Biochem. Biophys. 165, 468—477
26 Pledger, W.J., Thompson, W.J. and Strada, S.J. (1975) Biochim. Biophys. Acta 391, 334—340
27 Gardner, E.A., Thompson, W.J., Stancel, G.M. and Strada, S.J. (1977) Fed. Proc. 36, 320
28 Gardner, E.A., Thompson, W.J., Strada, S.J. and Stancel, G.M. (1978) Biochemistry 17, 2995—3000
29 Patterson, W.D., Hardman, J.G. and Sutherland, E.W. (1976) Mol. Cell. Endocrinol. 5, 51—66
30 Russell, T.R., Terasaki, W.L. and Appleman, M.M. (1973) J. Biol. Chem. 248, 1334—1340
31 Bergstrand, H. and Lundquist, B. (1976) Biochemistry 15, 1727—1735
32 Terai, M., Furihata, C., Matsushima, T. and Sugimura, T. (1976) Arch. Biochem. Biophys. 176, 621—629
33 Miki, N. and Yoshida, H. (1972) Biochim. Biophys. Acta 268, 166—174
34 Wang, J.H. and Desai, R. (1977) J. Biol. Chem. 252, 4175—4184
35 Takai, Y., Yamamoto, M., Inoue, M., Kishimoto, A. and Nishizuka, Y. (1977) Biochem. Biophys. Res. Commun. 77, 542—550
36 Smoake, J.A., Song, S.-Y. and Cheung, W.Y. (1974) Biochim. Biophys. Acta 341, 402—411
37 Weiss, B., Fertel, R., Figlin, R. and Uzunov, P. (1974) Mol. Pharmacol. 10, 615—625
38 Goren, E.N. and Rosen, O.M. (1971) Arch. Biochem. Biophys. 142, 720—723
39 Appleman, M.M. and Terasaki, W.L. (1975) Adv. Cyclic Nucl. Res. 5, 153—162
40 Teo, T.S., Wang, T.H. and Wang, J.H. (1973) J. Biol. Chem. 248, 588—595
41 Brostrom, C.O. and Wolff, D.J. (1976) Arch. Biochem. Biophys. 172, 301—311
42 Wolff, D.J. and Brostrom, C.O. (1976) Arch. Biochem. Biophys. 173, 720—741
43 Pichard, A.-L. and Cheung., W.Y. (1977) J. Biol. Chem. 252, 4872—4875
44 Cheung, W.Y. and Lin, Y.M. (1974) Methods Enzymol. 38, 223—239
45 Mills, J.S., Epstein, P.M., Strada, S.J. and Thomspon, W.J. (1978) Adv. Cyclic Nucl. Res. 9, 758
46 Van Inwegen, R.G., Pledger, W.J., Strada, S.J. and Thompson, W.J. (1976) Arch. Biochem. Biophys. 175, 700—709
47 Neurath, H. and Walsh, K.A. (1976) Proc. Natl. Acad. Sci. U.S. 73, 3825—3832
48 Lazo, P., Sun, S. and Tsolas, O. (1977) Fed. Proc. 36, 776
49 Cohen, P. (1973) Eur. J. Biochem. 34, 1—14
50 Wallach, D., Anderson, W. and Pastan, I. (1978) J. Biol. Chem. 253, 24—26
51 DeMoss, J.A. (1977) J. Biol. Chem. 252, 1696—1701
52 Brakel, C.L. and Blumenthal, A.B. (1977) Biochemistry 16, 3137—3143
53 Carlson, K.E., Sun, J.-H.K. and Katzenellenbogen, J.A. (1977) Biochemistry 16, 4288—4296