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Epiphyseal Cartilage Cyclic Nucleotide Phosphodiesterase: Partial Purification and Kinetic Characteristics of Multiple Enzymes (39338)

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Multiple forms of 3′,5′-cyclic AMP phosphodiesterase (PDE) have been demonstrated in many tissues (1–13), and these enzymes may have important and different regulatory functions within cells. Previous work from our laboratory has identified cyclic AMP PDE activity in chicken epiphyseal and articular cartilage (14). We have also demonstrated the inhibition of the low K_m species of these enzymes by L-triiodothyronine (T-3) and several nonsteroidal anti-inflammatory agents (14, 15).

To determine the physiological significance of PDE inhibition by these pharmacological agents and to relate the inhibition to cell regulatory mechanisms, it was necessary to characterize the multiple molecular forms of PDE present in cartilage. In this paper, we describe the presence of multiple molecular forms of PDE in epiphyseal cartilage, differing by their affinity for cyclic AMP and cyclic GMP, their inhibition by triiodothyronine (T-3) and indomethacin, and their separation on DEAE Bio-Gel A column chromatography.

Materials and methods. Ammonium salts of cyclic [G-^{3}H]AMP (24.0 Ci/mmole) and of cyclic [G-^{3}H]GMP (3.46 Ci/mmole) were purchased from New England Nuclear. The radioactive compounds were purified by thin-layer chromatography before use as follows. A 50% ethanol solution of either cyclic nucleotide was applied to an Eastman chromatogram cellulose sheet (13255) as a streak (10–15 μl per cm), and the chromatograph was developed with isopropanol–conc. NH_4OH–water (7:1:2, v/v) for 4 hr. The R_f values for cyclic AMP and cyclic GMP in this system were 0.56–0.60 and 0.32–0.36, respectively. After drying the chromatogram, the cyclic nucleotide zone was scraped off, and the nucleotide extracted with water at 5°C for 2 to 3 hr. After centrifugation to clear the radioactive solution, the cyclic nucleotide solutions were stored at −20°.

Snake venom *(Ophiophagus hannah)* and cyclic GMP were obtained from Sigma, and cyclic AMP (free acid, monohydrate) was from P-L Biochemicals, Inc. DEAE Bio-Gel A (100–200 mesh) was obtained from Bio-Rad Laboratories and was equilibrated with the column buffer prior to use. Anion exchange resin (Bio-Rad AG1-X2, 200 to 400 mesh) was washed in succession with 0.5 *N* NaOH, H_2O, 0.5 *N* HCl and H_2O until neutral. Indomethacin was provided by Dr. T. Y. Shen of the Merck Institute for Therapeutic Research, and T-3 (3,3′,5-triiodo-L-thyronine) was obtained from Schwarz/Mann.

Phosphodiesterase activity was assayed by a procedure modified from the method of Thompson and Appleman (16, 17). The reaction mixture was identical to that described by them (16, 17) except that it contained 0.1 ml of snake venom. Reactions were initiated by addition of the PDE preparation, and the mixture was incubated for 10 min at 30°. Reactions were stopped by the addition of 0.5 ml of cold absolute ethanol and chilled in an ice-water bath. The reaction mixture was then transferred onto a small anion exchange column (5 × 30 mm) which was prepared from 1.5 ml of 1:2 slurry of AG1-X2 resin in a Pasteur pipet (7 × 146 mm). The reaction tube and the column were washed successively with 3 × 1 ml of water, allowing each wash to sink into the resin before adding the next one. An aliquot of 1 ml out of 4 ml of the combined effluent and washings was counted in 10 ml of scintillation fluid. Enzyme activity was linear with respect to time, protein concentration, and up to 20% conversion of substrate to product under the conditions of the assay as described. Counting of ^{3}H was carried out with the Model 3310 Packard Tri-

Carb liquid scintillation spectrometer. The scintillant solution was a modified Bray's solution (18) which contained 60 g of naphthalene, 4 g of 2,5-diphenyloxazole, 200 mg of 1,4-di-2-(5-phenyloxazolyl) benzene in 100 ml of methanol, and 900 ml of 1,4-dioxane. When counted in 10 ml of liquid scintilator, the efficiency of this method for tritium was 20% in the presence of 1.0 ml of tritiated water.

One unit of PDE activity was defined as the amount of enzyme required to hydrolyze 1 pmole of cyclic mononucleotide per min at 30°. Specific activity was expressed as enzyme units per milligram of protein.

Upper epiphyseal cartilage zones removed from 8-week-old hybrid Hubbard broiler chickens by the method of Wuthier (19) as previously described (14) were used as the enzyme source. The fine shavings from four chickens were homogenized in 48 ml of ice-cold 0.05 *M* Tris–Cl buffer (pH 8.0) containing 0.25 *M* sucrose, and then centrifuged at 100,000 *g* for 90 min. The slightly reddish clear supernate solution, containing approx 2.7 mg of protein per ml, was used as the source of the crude soluble enzyme. The precipitate from the 100,000 *g* centrifugation was rehomogenized and centrifuged at 100,000 *g* thrice more, and finally suspended in the homogenizing solution to a volume equal to one-third the first supernatant solution. The soluble and particulate fraction could be stored at −20° for at least several weeks without loss of activity. Protein determinations were carried out by the Lowry procedure (20) with the use of crystalline bovine serum albumin as a standard. For column fractions in which 2-mercaptoethanol was present, a standard curve was prepared in the presence of the column buffer.

The DEAE Bio-Gel A column (1.3 × 22 cm) used for enzyme purification was equilibrated with 0.05 *M* Tris–Cl buffer (pH 8.0) containing 3.75 m*M* 2-mercaptoethanol. A portion of the soluble enzyme (30 mg of protein) dialyzed overnight against the column buffer, was applied to the column, and then washed with the same buffer until the A_{280} in the washing returned to a base line level. The column was then eluted with a linear gradient generated from 150 ml of the column buffer and 150 ml of the buffer containing 0.4 *M* NaCl, and 5-ml fractions were collected. The column flow rate was approx 12 ml/hr. One-tenth milliliter of the enzyme solution was used to assay for cyclic AMP PDE activity and 0.2 ml for cyclic GMP PDE activity. Substrate concentration was 1 μM in both cases.

Enzyme control incubations included a no enzyme reaction mixture, appropriate solvent concentrations (2% v/v DMSO) to account for any inhibition due to the solvent used with triiodothyronine and indomethacin and incubations to establish that the observed inhibition of PDE activity was not the result of the inhibition of 5′-nucleotidase activity. The assay for 5′-nucleotidase activity was performed using the same reaction mixture as described for the PDE assay except for the use of 5′-AMP as the substrate instead of cyclic AMP and snake venom was omitted. I_{50} values were obtained by plotting the logarithm of inhibitor concentration as a function of the percentage of enzyme inhibition. To determine the apparent K_m and V of the partially purified enzymes, substrate concentrations between 0.8 and 16 μM were used and the specific radioactivities of the substrate, cyclic [^{3}H]AMP, and cyclic [^{3}H]GMP, were 52,500 cpm/nmole and 47,500 cpm/nmole, respectively. Nonlinear Lineweaver-Burk plots for chicken epiphyseal cartilage PDE have been shown previously (14); the present investigations only defined low K_m values since they are probably more important with respect to the physiological activity of the enzyme.

Results. Separation of cyclic 3′,5′-nucleotide phosphodiesterase activities by column chromatography. The high speed supernatant solution of chicken epiphyseal cartilage extract was fractionated by DEAE Bio-Gel A column chromatography, and the fractions were analyzed for cyclic mononucleotide hydrolyzing activity, 5′-nucleotidase activity, and protein content (Fig. 1).

Despite the use of a different ion exchange column (DEAE-cellulose) and various elution procedures, the cyclic AMP PDE activities in the major peak (II_s and II) could not be resolved into two separate peaks. As shown in Fig. 1, peak I utilized both cyclic AMP and GMP as substrates; the activity towards cyclic GMP was much

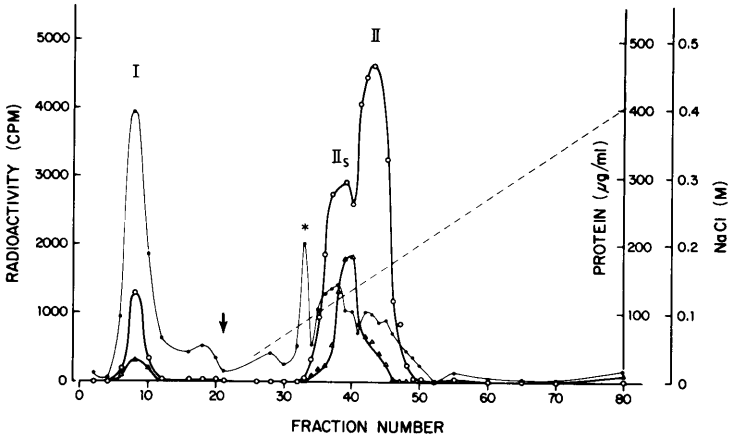

FIG. 1. Separation of multiple forms of PDE from chicken cartilage extract. Unabsorbed protein was washed off and then (↓) a linear NaCl gradient (- - -) up to 0.4 *M* was applied. Fractions of 5 ml were collected and their cyclic AMP-PDE (○) and cyclic GMP-PDE (△) activities as well as their protein content (●) were monitored. Radioactivities found in 1-ml aliquots from 4-ml filtrate (see the text) were shown. The nucleotidase-activity peak was found in fraction 33 (*).

lower than that for cyclic AMP. The major peak appeared also to utilize both cyclic nucleotides as substrates; however, the enzyme activity towards cyclic GMP disappeared from the fractions obtained after the maximal cyclic AMP-PDE activity was attained. These findings suggested that two forms of PDE existed, one associated with the shoulder (II_s) which utilized both cyclic nucleotides, and the other associated with the slowest eluting peak (II) which utilized only cyclic AMP as substrate. As compared to the specific activity of the crude epiphyseal enzyme with cyclic AMP as the substrate, peak I specific activity was increased by a factor of 1.5, the apex of the peak II_s was increased by 10.5 and peak II (fractions from the downslope) by 20-fold. The peak denoted by the asterisk (Fig. 1) contained all the 5′-nucleotidase activity present in the column eluant.

Kinetic characteristics of the PDE chromatographic fractions. Peak I, which represented a single homogeneous peak, had an apparent K_m of 5 μM with cyclic AMP as substrate and a V of 36 units ml^{-1}. The low activity of this enzyme with cyclic GMP as substrate obviated the determination of these kinetic parameters with that substrate. Peak II_s had an apparent K_m of 1.2 μM with cyclic AMP as substrate and a V of 38 units ml^{-1}. Peak II, using fractions without cyclic GMP activity, had an apparent K_m of 1.2 μM and a V of 45 units ml^{-1}. Kinetic analyses of the fraction with highest activity towards cyclic GMP in peak II_s gave an apparent K_m of 1.2 μM for cyclic GMP as substrate and a V of 29 units ml^{-1}.

TABLE I. THE EFFECT OF INDOMETHACIN AND L-TRIIODOTHYRONINE ON PURIFIED PDE FRACTIONS.[a]

Chromatographic fraction	I_{50} values (M)	
	Indomethacin	T-3
Peak I	1.7×10^{-3}	1.8×10^{-4}
Peak II_s	2.5×10^{-5}	1.0×10^{-5}
Peak II	1.1×10^{-4}	3.0×10^{-6}

[a] I_{50} values were obtained using 1 μM cyclic AMP as the substrate.

Inhibition of purified PDE by indomethacin and L-triiodothyronine. Varying the inhibitor concentrations and determining the percentage of normal (no inhibitor) enzyme activities in a soluble and particulate enzyme preparation gave relatively high I_{50} values and demonstrated no particular preference for the inhibition of the soluble or particulate enzyme species by indomethacin or T-3. Under these conditions, the I_{50} values for these inhibitors showed relatively similar capacities to inhibit these enzyme species.

The effect of these inhibitors on various soluble enzyme fractions purified by column chromatography are shown in Table I. A

comparison of the I_{50} for indomethacin and T-3 obtained from various enzyme fractions after DEAE Bio-Gel A chromatography demonstrated differences in the capacity of indomethacin and T-3 to inhibit these enzyme fractions.

Discussion. Since work from our laboratory has shown that triiodothyronine (T-3), the most potent thyroid hormone, and indomethacin, a moderately potent anti-inflammatory agent inhibit crude epiphyseal cartilage cyclic nucleotide phosphodiesterase, the present studies were undertaken to purify epiphyseal cartilage PDE and to test various purified fractions for their inhibition by these agents.

The modified PDE assay, as described herein, avoided the high values for the enzyme blanks caused by the heat step in the original Appleman assay (16, 17). The use of cold ethanol to terminate the one-step assay is a simple and adequate method and is somewhat easier to perform than the method proposed by Appleman for his revised assay (21). An additional difficulty was encountered when the original PDE assay (16, 17) was used to assay purified fractions after ion exchange chromatography. The presence of sodium acetate or sodium chloride resulted in an increase in the enzyme blank. This is caused by the diminished capacity of the batch treatment of the reaction mixture with anion exchange resin to remove PDE substrates. The modified assay did not give high blank values in the presence of salts, had a low blank value, and permitted the assay of PDE activity at low enzyme concentrations. A further advantage of this assay on the PDE isolated from epiphyseal cartilage was the presence of significant 5′-nucleotidase activity in the crude enzyme. Approximately 90% of the 5′-adenosine monophosphate formed in the reaction was converted to adenosine by 0.3 mg of crude cartilage enzyme in the absence of snake venom (5′-nucleotidase). Thus, a one-step assay with 5′-nucleotidase in the reaction mixture from the outset was more advantageous for our work than the two-step Appleman procedure.

Analyses of a carefully washed 100,000 *g* sedimented enzyme fraction demonstrated PDE activity; therefore as has been shown for other tissues (22), epiphyseal cartilage contained a particulate and soluble PDE fraction. The particulate enzyme utilized both cyclic AMP and GMP as substrates. The relative percentages of PDE activity in the soluble and particulate fractions with 1 μM cyclic AMP as substrate were 86.5 and 13.5%, respectively. With cyclic GMP (1 μM) as substrate the relative percentage of the soluble and particulate fractions were 93.5 and 6.5%, respectively.

The I_{50} values determined for indomethacin and T-3 showed that peak I was inhibited by these agents at relatively high concentrations (Table I). These high values probably indicate that no physiological significance should be given to the effect of these agents on peak I enzyme activity. The ratio of the I_{50} values obtained with indomethacin and enzyme fractions from peaks II_s and II showed a 4.4-fold difference. Similar data were obtained by comparing T-3 I_{50} values from peak II_s and II. Since the I_{50} value for T-3 was lowest with peak II fractions and the I_{50} value for indomethacin was lowest for peak II_s, it might be suggested that there is a difference in drug sensitivity between the enzyme activities of II and II_s.

Although by no means conclusive proof, the substrate specificities and the inhibitor activities suggest that the major PDE peak (II_s and II) probably represents two separate enzyme species. The literature supports such a conclusion since many workers have now described three peaks of PDE activity in various tissues (17, 23–26).

The concentration of indomethacin resulting in PDE inhibition is within the dosage range of this drug in clinical practice. The fact that this agent is an inhibitor of a low K_m species of PDE, the proposed physiologically active enzyme, permits one to consider the pharmacological effect(s) of this anti-inflammatory drug on cyclic nucleotide metabolism in epiphyseal cartilage.

Even though the concentrations of T-3 inhibiting PDE activity are not within the physiological range, we (15) and others (24–35) have addressed this question of comparing *in vitro* hormone effects with *in vivo* action. Since thyroid hormone does effect bone growth and maturation *in vivo*, the effect of T-3 on the enzymes involved in

cyclic nucleotide catabolism suggests to us that one of the regulatory effects of this hormone may be mediated via the cyclic nucleotide system.

Since PDE activity probably has a role in the regulation of cell growth and proliferation (36), manipulation of specific PDE enzyme(s) by such inhibitors may represent an approach to altering certain pathological processes such as premature epiphyseal closure. Subsequent studies may provide correlations between cell structure and enzyme function.

From a basic point of view, the PDE inhibitors, triiodothyronine and indomethacin, may be useful in the investigation of PDE heterogeneity in other tissues.

Summary. Partial purification of chicken epiphyseal PDE activity by centrifugation and column chromatography has defined two distinct peaks of PDE activity. The faster eluting peak (I) has a higher apparent K_m for cyclic AMP than the slower eluting major peak (II_s and II). Peak I has greater activity towards cyclic AMP as a substrate than towards cyclic GMP but does use both substrates. Peak I is not inhibited by T-3 or indomethacin at physiological concentrations. Substrates studies demonstrate the presence of at least two overlapping PDE species in the major peak (II_s and II). There is suggestive evidence that indomethacin is a more potent inhibitor of peak II_s which can use either cyclic AMP or cyclic GMP as substrates, whereas T-3 is a more potent inhibitor of fractions eluting where the enzyme only has activity with cyclic AMP.

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1. Brooker, G., Thomas, L. J., Jr., and Appleman, M. M., Biochemistry **7,** 4177 (1968).
2. Cheung, W. Y., Adv. Biochem. Psychopharmacol. **3,** 51 (1970).
3. Hardman, J. G., Robison, G. A., and Sutherland, E. W., Annu. Rev. Physiol. **33,** 311 (1971).
4. Thompson, W. J., and Appleman, M. M., Ann. N.Y. Acad. Sci. **185,** 36 (1971).
5. Thompson, W. J., and Appleman, M. M., J. Biol. Chem., **246,** 3145 (1971).
6. Goren, E. N., Hirsch, A. H., and Rosen, O. M., Anal. Biochem. **43,** 156 (1971).
7. Monn, E., and Christiansen, R. O., Science (Washington, D.C.) **173,** 540 (1971).
8. Monn, E., Desautel, M., and Christiansen, R. O., Endocrinology **91,** 716 (1972).
9. Pichard, A.-L., Hanoune, J., and Kaplan, J.-C., Biochim. Biophys. Acta **279,** 217 (1972).
10. Pichard, A.-L., Hanoune, J., and Kaplan, J.-C., Biochim. Biophys. Acta **315,** 370 (1973).
11. Klotz, U., Berndt, S., and Stock, K., Life Sci. **11** (Part II), 7 (1972).
12. Goren, E. N., and Rosen, O. M., Arch. Biochem. Biophys. **153,** 384 (1972).
13. Russell, T. R., Thompson, W. J., Schneider, F. W., and Appleman, M. M., Proc. Natl. Acad. Sci. USA **69,** 1791 (1972).
14. Newcombe, D. S., Thanassi, N. M., and Ciosek, C. P., Jr., Life Sci. **14,** 505 (1974).
15. Thanassi, N. M., and Newcombe, D. S., Proc. Soc. Exp. Biol. Med. **147,** 710 (1974).
16. Thompson, W. J., and Appleman, M. M., Biochemistry **10,** 311 (1971).
17. Russell, T. R., Terasaki, W. L., and Appleman, M. M., J. Biol. Chem. **248,** 1334 (1973).
18. Bray, G. A., Anal. Biochem. **1,** 279 (1960).
19. Wuthier, R. E., Calcif. Tissue Res. **4,** 20 (1969).
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
21. Thompson, W. J., Brooker, G., and Appleman, M. M., *in* "Methods in Enzymology" (J. G. Hardman and B. W. O'Malley, eds.), Vol. 38 (Part C), p. 205. Academic Press, New York (1974).
22. Appleman, M. M., Thompson, W. J., and Russell, T. R., *in* "Advances in Cyclic Nucleotide Research" (P. Greengard, and G. A. Robison, eds.), Vol. 3, p. 76. Raven Press, New York (1973).
23. Appleman, M. M., and Terasaki, W. L., *in* "Advances in Cyclic Nucleotide Research" (G. I. Drummond, P. Greengard, and G. A. Robison, eds.), Vol. 5, p. 153. Raven Press, New York (1975).
24. Kakiuchi, S., Yamazaki, R., Teshima, Y., Ueneshi, K., and Miyamoto, E., *in* "Advances in Cyclic Nucleotide Research" (G. I. Drummond, P. Greengard, and G. A. Robison, eds.), Vol. 5, p. 163. Raven Press, New York (1975).
25. Wang, J. H., Teo, T. S., Ho, H. C., and Stevens, F. C., *in* "Advances in Cyclic Nucleotide Research" (G. I. Drummond, P. Greengard, and G. A. Robison, eds.), Vol. 5, p. 179. Raven Press, New York (1975).
26. Weiss, B., *in* "Advances in Cyclic Nucleotide Research" (G. I. Drummond, P. Greengard, and G. A. Robison, eds.), Vol. 5, p. 195. Raven Press, New York (1975).
27. Levey, G. S., and Epstein, S. E., J. Clin. Invest. **48,** 1663 (1969).
28. Levey, G. S., Recent Progr. Horm. Res. **29,** 361 (1973).

29. Tapley, D. F., and Cooper, C., J. Biol. Chem. **222,** 341 (1956).
30. Maley, G. F., and Lardy, H. A., J. Biol. Chem. **204,** 435 (1953).
31. Dunne, P. B., and Tapley, D. F., Nature (London) **185,** 622 (1960).
32. Sokoloff, L., and Kaufman, S., J. Biol. Chem. **236,** 795 (1961).
33. Tapley, D. F., J. Biol. Chem. **222,** 325 (1956).
34. Pittman, J. A., Tingley, J. O., Nickerson, J. F., and Hill, S. R., Jr., Metab. Clin. Exp. **9,** 293 (1960).
35. Klebanoff, S. J., J. Biol. Chem. **234,** 2437 (1959).
36. Pastan, I. H., Johnson, G. S., and Anderson, W. B., Annu. Rev. Biochem. **44,** 491 (1975).

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