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Chromatographic Behavior of Cyclic 3′,5′-Nucleotide Phosphodiesterases on Columns of Immobilized Inhibitors†

Anish Mohindru, Alix Chenet, and Allen R. Rhoads*

ABSTRACT: The chromatographic behavior of cyclic 3′,5′-nucleotide phosphodiesterase from bovine heart ventricle was studied on columns of immobilized inhibitors. Succinylated trimethylpapaveroline (STMP) and the 7-acetic acid derivative of 1-methyl-3-isobutylxanthine (MIX) were coupled to diaminodipropylamine-substituted agarose. Chromatography was performed either in the presence or absence of calcium. In the presence of 100 μM calcium, the heart enzyme eluted as a single peak of activity from the STMP-agarose column. When the calcium in the elution buffer was replaced by 100 μM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), phosphodiesterase was resolved by chromatography on STMP-agarose into two forms of activity. The endogenous protein activator of heart phosphodiesterase eluted between the two activity peaks. The first activity peak (peak I) could be stimulated several fold by the addition of a calcium-dependent protein activator of bovine brain cortex. This activator-dependent form of phosphodiesterase was also subject to activation by proteolytic treatment. In contrast, the second activity peak (peak II) did not respond to protein activator or to proteolytic treatment. Peak II was more sensitive to inhibition by papaverine than peak I. Additional evidence from kinetic studies, sensitivity to sulfhydryl reagents, and polyacrylamide gel electrophoresis indicate that peaks I and II are distinct forms of phosphodiesterase activity. When phosphodiesterase of heart was chromatographed on MIX-agarose in the presence of calcium or EGTA, the elution profile was similar to that observed on STMP-agarose. Two activity peaks differing in their response to the protein activator were again resolved in the presence of EGTA. The performance of the substituted agaroses in separating phosphodiesterase of bovine cerebrum was also examined and compared with results of heart tissue.

The propensity of cyclic 3′,5′-nucleotide phosphodiesterase (EC 3.1.4.17) to exhibit multiple molecular forms in many mammalian tissues has been repeatedly observed by many laboratories (Thompson and Appleman, 1971a; Monn and Christiansen, 1971; Kakiuchi et al., 1971; Uzunov and Weiss, 1972; Russell et al., 1973). This multiplicity of enzymes may be due to both their localization in several subcellular fractions (Beavo et al., 1970; Cheung and Salganicoff, 1967) and to recent findings indicating subunit heterogeneity among the phosphodiesterases (Cheung, 1970, 1971; Kakiuchi et al. 1971). Both Cheung (1970, 1971) and Kakiuchi et al. (1971) demonstrated that certain phosphodiesterases have a calcium-dependent protein activator, which dissociates from the enzyme during purification. This heat-stable, calcium-binding protein activator from brain (Lin et al., 1974) and heart (Teo and Wang, 1973) has been purified to homogeneity and extensively characterized. This activator subunit is not associated with all phosphodiesterases of the same tissue (Uzunov and Weiss, 1972; Weiss, 1975; Pledger et al., 1975). These findings suggest subunit heterogeneity among phosphodiesterases, but knowledge of the precise subunit composition must await the homogeneous purification of these enzymes. The reported differences in the sensitivity of multiple forms of phosphodiesterase to inhibition by alkylated xanthine and papaverine

† From the Department of Biochemistry, Howard University College of Medicine, Washington, D.C. 20059. *Received November 16, 1977; revised manuscript received April 3, 1978.* This work was supported by a grant (BMS 75-16751) from the National Science Foundation. A preliminary report of this work has been presented (Mohindru and Rhoads, 1977).

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(Uzunov and Weiss, 1975; Weiss et al., 1974; Wells et al., 1975; Hidaka et al., 1975) and the need of multiple separation techniques in the characterization of this enzyme system (Van Inwegen, 1976) led us to investigate the chromatographic behavior of phosphodiesterases from several tissues on columns of immobilized inhibitors. In addition to the selective isolation and purification of enzymes (Cuatrecasas, 1970), affinity chromatography has proven useful in the separation of isozymes (Brodelius and Mosbach, 1973; O'Carra and Barry, 1974). The ability of this technique to resolve the multiple forms of phosphodiesterase was of particular interest in this study.

Materials and Methods

Materials. The ^{3}H-labeled nucleotides, cyclic [8-^{3}H]AMP[1] (sp act. 20 Ci/mmol) and cyclic [8-^{3}H]GMP (sp act. 8.8 Ci/mmol), were purified on DEAE-Sephadex (A-25) prior to use. Theophylline, 1-methyl-3-isobutylxanthine, and trimethylpapaveroline hydrochloride were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. All other chemicals and enzymatic reagents were purchased from Sigma Chemical Co., St. Louis, Mo. Sepharose 4B and Sephadex gels were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of Diaminodipropylamine-Substituted Sepharose 4B. The gel was prepared as described by Cuatrecasas (1970). Forty milliliters of Sepharose 4B (settled volume) was reacted with 8.0 g of CNBr in H_2O and maintained at pH 11 and at 20 °C. After washing with 12 volumes of 0.1 M $NaHCO_3$ (pH 10.0), the activated gel was immediately reacted with 80 mmol of 3,3′-iminobis(propylamine) in 40 mL of 0.1 M bicarbonate buffer at pH 10.0. The suspension was stirred slowly at 4 °C for 16 h. The diaminodipropylamine-substituted Sepharose was washed extensively with distilled water to remove uncoupled amine

Preparation of the 7-Acetic Acid Derivative of 1-Methyl-3-isobutylxanthine. 1-Methyl-3-isobutylxanthine (13.5 mmol) in 30 mL of distilled water containing 27 mmol of NaOH was reacted with 13.5 mmol of $ClCH_2COOH$ in 20 mL of distilled water containing equimolar NaOH. The reaction mixture was gently refluxed for 4 h and kept at room temperature for 24 h. After adjusting the pH to 8.0 with dilute HCl, the unreacted xanthine was removed by filtration through a sintered glass funnel. The acetic acid derivative was precipitated by lowering the pH to below 1.0 with 6 N HCl. The yield of free acid ranged from 45 to 55%. The derivative was recrystallized twice from boiling ethanol. The melting point was 187–190 °C. Chromatography of the derivative on 0.1-mm polyethyleneiminecellulose thin-layers developed in 65% (v/v) ethanol gave a R_f of 0.79 with no impurities under ultraviolet light. The R_f of the parent compound in this system was 0.96. The structure and composition of the derivative was confirmed by infrared spectroscopy and nitrogen analysis.

Preparation of Succinyltrimethylpapaveroline. Trimethylpapaveroline hydrochloride (1.4 mmol) was dissolved in 15 mL of anhydrous dimethylformamide and reacted with 2.77 mmol of succinic anhydride. The reaction mixture was stirred at room temperature for 18 h. The mixture was concentrated to approximately 5 mL, and 20 mL of distilled water was added. Titration of the aqueous solution to pH 4.4 with 1 N NaOH precipitated the succinyl derivative as pale-yellow crystals. The acid derivative had a sharp melting point of 135 °C (parent compounds, 255 °C). Thin-layer chromatography of the acid derivative and trimethylpapaveroline on silicic acid developed with benzene–methanol–acetic acid (9:10:1, v/v) gave R_f values of 0.37 and 0.53, respectively. Structure was confirmed by infrared spectroscopy.

Preparation of Ligand-Substituted Gels. The 1-methyl-3-isobutyl-7-xanthine acetic acid substituted gel was prepared by dissolving 2.5 mmol of the acetic acid derivative of the alkylated xanthine in 50 mL of 20% (v/v) dimethylformamide containing 25 mL of diaminodipropylamine-substituted Sepharose (settled volume). The pH of the reaction was brought to 4.8 with 1 N HCl, and 2.63 g of EDC in 2.5 mL of water was added. The reaction mixture was slowly stirred at 25 °C for 24 h. The ligand-substituted gel was washed with distilled water. Unbound ligand in the washes was determined by ultraviolet spectroscopy. Substitution occurred to the extent of 18.5 μmol of 1-methyl-3-isobutyl-7-xanthineacetic acid per mL of settled gel.

The succinyl derivative of trimethylpapaveroline was covalently linked to the diaminodipropylamine-substituted Sepharose as follows: 638 mg (1.5 mmol) of the succinylated trimethylpapaveroline was dissolved in 30 mL of 50% (v/v) dioxane and added to 25 mL (settled volume) of the diaminodipropylamine-substituted Sepharose in 25 mL of water. EDC (1.5 g) was added in 4.0 mL of water. The pH was brought to 4.75 and the reaction allowed to proceed for 5 h. Ultraviolet determination of unbound ligand indicated that 21 μmol of the acid derivative was bound per mL of settled gel.

Preparation of Phosphodiesterases Prior to Chromatography. Beef heart and brain obtained from Frederick County Products, Inc., Frederick, Md., were stored at −20 °C until preparation of the enzymes. The ventricles were trimmed of fat and connective tissue and minced in a meat grinder. The tissue was homogenized in a Waring blender for 2 min with 3 volumes of 0.25 M sucrose containing 1 mM $MgCl_2$/g of tissue. After centrifugation at 10 500*g* for 20 min, the supernatant was collected and poured through several layers of cheesecloth. The pH was adjusted to 7.4 with 2 N NaOH. Solid $(NH_4)_2SO_4$ was added to 55% saturation, and the solution was stirred for 30 min. After centrifugation at 10 500*g* for 20 min, the pellet was resuspended in 12 mM imidazole buffer containing 1 mM $MgCl_2$, 0.5 mM dithiothreitol at pH 7.4. The suspension was centrifuged at 12 000*g* for 10 min and desalted on a Sephadex G-25 column (2.6 × 25 cm). This enzyme preparation was lyophilized and stored at −20 °C. Before chromatography on the immobilized inhibitor columns, the enzyme was equilibrated with starting buffer on a Sephadex G-25 column (2.6 × 25 cm). The specific activity of this enzyme preparation ranged from 50 to 60 milliunits/mg of protein, as measured by orthophosphate release.

The enzyme from brain was prepared from bovine cortices according to the method of Cheung (1969). The $(NH_4)_2SO_4$ pellet was resuspended in imidazole buffer and prepared in the same manner as described for the heart enzyme.

Preparation of Bovine Brain Protein Activator. The protein activator of phosphodiesterase was prepared from bovine brain cerebrum through the heat treatment step according to the method of Lin et al. (1974). After heat treatment, the supernatant was concentrated by lyophilization, resuspended in 25 mM Tris-HCl, pH 7.4, containing 0.5 mM dithiothreitol and 1.0 mM $MgCl_2$ (buffer A) and desalted on Sephadex G-25. This preparation of the protein activator was stored frozen in small batches and used in all subsequent analyses to determine the effect of activator on enzyme activity. Maximum stimu-

[1] Abbreviations used are: cyclic AMP, cyclic adenosine 3′,5′-monophosphate; cyclic GMP, cyclic guanosine 3′,5′-monophosphate; R_f, mobility of compound relative to solvent front; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EGTA, ethylenebis(oxyethylenentrilo)tetraacetic acid; K_M, Michaelis-Menten constant; I_{50}, concentration necessary for 50% inhibition.

lation was determined by construction of a dose–response curve for each enzyme preparation.

Chromatography Procedures. The inhibitor-substituted gels were packed into columns with dimensions of 1.6 × 3.0 cm (xanthine-substituted agarose) or 1.6 × 6.0 cm (succinyltrimethylpapaveroline-substituted agarose). The columns were then washed and equilibrated with buffer A. Elution buffers consisted of buffer A supplemented with either 100 μM $CaCl_2$ or 100 μM EGTA. The amount of protein applied to the column ranged from 7 to 64 mg. Samples in excess of 300 mg of protein were sufficient to overload the STMP–agarose column and produce a loss in the resolution of the heart enzymes. After the sample was applied, the columns were washed with 90 to 100 mL of buffer A. The columns were then eluted with linear salt gradients in buffer A (200–300 mL) starting without salt and ending at 0.1 to 0.4 M NaCl. Gradient elution was followed in most experiments by washing the column with 0.15 M (MIX–agarose) or 1.0 M NaCl (STMP–agarose) in buffer A. The columns were eluted at a flow rate of 35–45 mL/h, and 3.0-mL fractions were collected.

Assay of Cyclic 3′5′-Nucleotide Phosphodiesterase. Cyclic 3′,5′-nucleotide phosphodiesterase activity was determined by three assay procedures.

Assay Procedure I. This procedure measured the release of inorganic phosphate after treatment of the primary reaction mixture with *Crotalus atrox* venom (Butcher and Sutherland, 1962). The reaction mixture contained 40 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 1 (heart enzyme) or 2 mM (brain enzyme) cyclic AMP in a final volume of 1.0 mL. Enzyme was added to the blank after 0.1 mL of 55% (w/v) trichloroacetic acid was added.

Assay Procedure II. This procedure was conducted according to the method of Thompson and Appleman (1971b) as modified by Londesborough (1976). Assay conditions were the same as described above, except that the final volume was 0.5 mL and 200 000 cpm of tritiated cyclic nucleotide was included with unlabeled substrate. The reaction mixture was incubated at 30 °C for 10 min and terminated by heating at 100 °C for 2 min. For kinetic determinations, substrate concentrations ranged from 1 to 100 μM and time of assay from 10 to 30 min with total substrate hydrolysis restricted to 20%. This primary reaction mixture was treated for an additional 10 min with 100 μg of *Crotalus atrox* venom. Activation produced by snake venom was measured at 1 μM cyclic AMP by including 100 μg of venom in the primary reaction mixture (single-step assay). A 1-mL aliquot of an aqueous alcohol slurry of Bio-Rad AG-1-X2 (30 g of dry resin weight, 34 mL of absolute ethanol, and water to give 100 mL of slurry) was then added to each tube with thorough mixing. After centrifugation, 0.5 mL of the supernatant was counted by liquid scintillation spectrometry at 44% efficiency. Spectrophotometric analysis of sample reaction mixtures demonstrated that 97% of the adenosine, 70% of the guanosine, and 84% of the inosine were recovered in the resin supernatant after centrifugation.

Assay Procedure III. The third method was a spectrophotometric assay for cyclic AMP phosphodiesterase as described by Drummond and Perrot-Yee (1961). Reaction conditions were the same as in the above procedures, except that 100 μg of *Crotalus atrox* venom and 0.4 unit of adenosine deaminase were included in the assay mixture to permit continuous monitoring of the reaction. Cyclic AMP concentration was 100 μM in a final volume of 1.0 mL. The rate of decrease in absorbance at 265 nm was recorded in semimicrocuvettes. In each assay, a milliunit of enzyme activity is defined as the production of 1 nmol of product/min at 30 °C.

Assay of Protein Activator. The endogenous protein activator was assayed after heat denaturation of fractions eluted from the substituted-agarose columns. After heating at 95 °C for 5 min to eliminate phosphodiesterase activity, the fractions were centrifuged at 2000*g* for 10 min. Aliquots of the supernatant were assayed for their ability to stimulate the activator-deficient phosphodiesterase separated on the same column. Basal and stimulated phosphodiesterase activity was measured by the radiometric assay at 1 μM cyclic AMP in the presence of 250 μM $CaCl_2$.

Analytical Polyacrylamide Gel Electrophoresis. Electrophoresis was performed according to Davis (1964) in 5 × 75 mm gel tubes. A 5% acrylamide separating gel (1.11 mL) containing 4 mM $MgCl_2$ and adjusted to pH 8.8 to 9.0 was polymerized, giving a gel length of approximately 6 cm. Before application of the stacking gel, separating gels were allowed to equilibrate overnight in 374 mM Tris-HCl (pH 8.5), containing 4 mM $MgCl_2$ and 1 mM dithiothreitol. The equilibrated gels were preelectrophoresed at 1 mA/gel for 15 min. A 0.1-mL aliquot of the standard stacking gel was polymerized over each separating gel. The sample (200 μL) was mixed with 25 μL of glycerol and 25 μL of 0.01% (w/v) bromphenol blue in 50% (v/v) glycerol and applied to the gel. The electrophoresis buffer contained 25 mM Tris-HCl, 192 mM glycine, 1 mM dithiothreitol, and 4 mM $MgCl_2$ at a pH of 8.5. During the first 30 min of electrophoresis, the current was set a 1.5 mA/gel. All operations were performed at 4 °C. Gels were removed from each tube and trimmed at the center of the bromphenol blue zone and at the interface with the stacking gel. The remaining gel was cut into 3-mm slices (13–15 fractions), and each slice was extracted in 0.3-mL of buffer A at 4 °C overnight. Each fraction was assayed for cyclic AMP phosphodiesterase activity at 0.1 μM cyclic AMP (assay procedure II) with a 30-min incubation time. The R_f values were expressed using bromphenol blue tracking dye as a reference.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Results

The partially purified phosphodiesterase preparation from heart ventricle was chromatographed on succinyltrimethylpapaveroline-substituted agarose (STMP–agarose). Figure 1 shows the activity profiles obtained in the presence of 100 μM $CaCl_2$ (left-hand panels). The elution profile was examined in the presence of EGTA because of previous reports (Wang et al., 1975; Donnelly, 1976) that the heart enzyme can be resolved into two peaks of activity on DEAE-cellulose when the calcium-specific chelating agent is included in the elution buffer. In the presence of calcium, the enzyme was bound to the column and eluted as a single peak of activity at 130 mM NaCl. Attempts to elute the enzyme using 0.5 mM papaverine in buffer A were unsuccessful. Cyclic GMP and cyclic AMP hydrolytic activity coincided on each activity scan. Pooling the high-activity fractions of this peak resulted in a 40% yield with a three- to fourfold purification. This enzyme was inhibited 48% by inclusion of 100 μM EGTA in the assay mixture when measured by orthophosphate release. The enzyme was not stimulated by the protein activator from bovine brain cortex. This would indicate that the protein activator was still associated with the enzyme. However, chromatography on STMP–agarose in the presence of 100 μM EGTA (Figure 1, right-hand panels) resolved ventricular phosphodiesterase into two well-separated peaks of activity (lower right-hand panel of Figure 1C). Peak I could be maximally activated (4.5-fold) by addition of 20 μg or more of brain protein activator when

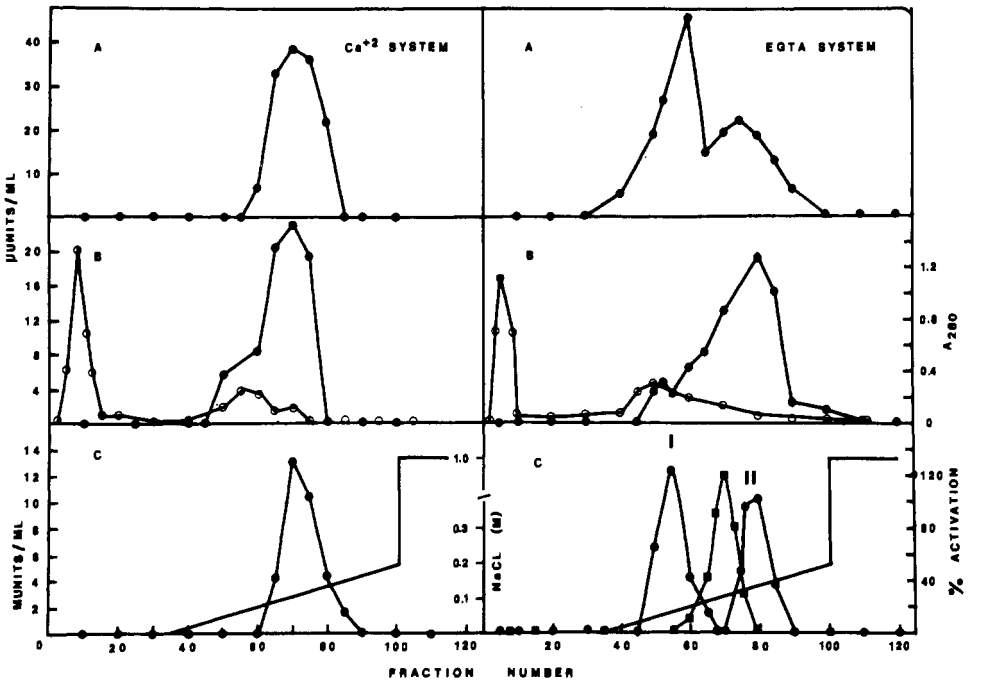

FIGURE 1: Chromatography of bovine heart phosphodiesterase on STMP-agarose. Protein (52.0 mg) was applied on a 1.6 × 6 cm column. Elution buffers contained either 100 μM $CaCl_2$ (left-hand panels) or 100 μM EGTA (right-hand panels). The column was washed with 100 mL of buffer A followed by a 200-mL linear gradient of NaCl in the same buffer. The final wash was with 1 M NaCl in buffer A. Other conditions are as described under Materials and Methods. Enzyme activity (●) was measured at the following substrate concentrations: panel A, activity was measured by radiometric assay at 0.1 μM cyclic GMP; panel B, activity was measured radiometrically at 0.1 μM cyclic AMP; panel C, enzyme activity was measured at 100 μM cyclic AMP by assay procedure III. Activator activity (■) is expressed as percent stimulation of pooled fractions from peak I by 250 μL of the heat-denatured fraction (see Materials and Methods). Absorbance (○) was measured at 280 nm.

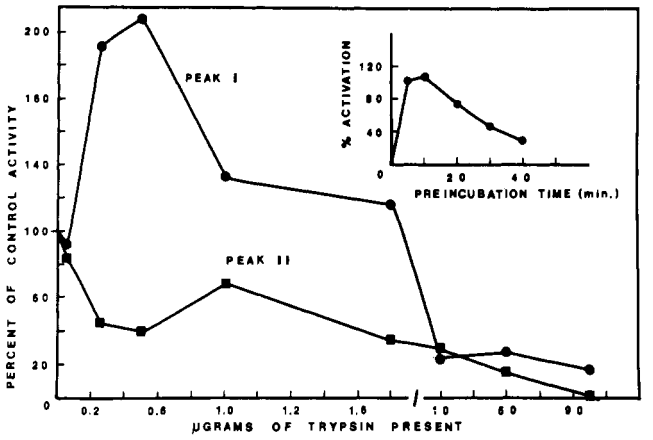

FIGURE 2: Effect of preincubation of isolated heart phosphodiesterases with trypsin. An aliquot of peaks I (16.5 μg of protein) and II (11.7 μg of protein) from the STMP-agarose column eluted in the presence of EGTA (Figure 1) was preincubated with increasing amounts of trypsin. Preincubation was performed in 67 mM Tris-HCl and 8.3 mM $MgCl_2$ at pH 7.4 in a total volume of 300 μL. After 10 min, the enzyme was assayed for activity at 1.0 μM cyclic AMP in the presence of excess soybean trypsin inhibitor (250 μg of protein) by the radiometric assay. The activity of both peaks I (○) and II (■) is expressed as percent of zero-time controls. Inset: peak I (16.5 μg of protein) was incubated with 500 ng of trypsin at the indicated time intervals under the same conditions.

TABLE I: Apparent Kinetic Constants for Bovine Heart Phosphodiesterases Obtained from STMP-Agarose Column.[a]

Enzyme	K_m (μM) substrate cAMP	K_m (μM) substrate cGMP	V_{max} (milliunits/mg) substrate cAMP	V_{max} (milliunits/mg) substrate cGMP
peak I				
nonact.	39	12	3.0	13
act.	30	7.0	7.0	30
peak II	10	8.0	15	12
Ca^{2+}-Complexed Enzyme	19	25	2.3	12

[a] All assays were performed by the radiometric method (procedure II). Under conditions similar to those described in Figure 1, pooled fractions of peaks I and II were obtained from STMP-agarose columns eluted in the presence of EGTA. The calcium-complexed enzyme was obtained from pooled fractions of the single peak eluted from STMP-agarose in the presence of $CaCl_2$ as described in Figure 1. Peak I was activated by the addition of 230 μg of brain protein activator to reaction mixture containing 250 μM $CaCl_2$.

measured at 1 μM cyclic AMP; however, under similar conditions peak II was unresponsive to the protein activator. The activator-responsive enzyme (peak I) was more active in the hydrolysis of cyclic GMP than cyclic AMP when measured at 0.1 μM (panels A and B of Figure 1). Peak II was equally active with either cyclic nucleotide as substrate.

In addition to the different response of peaks I and II to protein activator and substrate, the two enzymes also responded differently to protease treatment. Peak I was activated in a time-dependent manner when snake venom was present in the assay mixture (spectrophotometric assay). Activation by snake venom has been reported for the activator-deficient form of phosphodiesterase from bovine brain (Cheung, 1969). This activation was due to proteolytic activity in the venom. Proteolytic activation of activator-deficient phosphodiesterase may be a common property of the enzymes from different tissues, since the heart enzyme also responds to tryptic treatment (Figure 2). In this experiment, peak I was stimulated over twofold by tryptic digestion before beginning a slow decline in activity as the amount of trypsin increased. This activation could be reproduced by preincubating peak I with a fixed amount of trypsin (Figure 2, inset). In contrast, peak II was

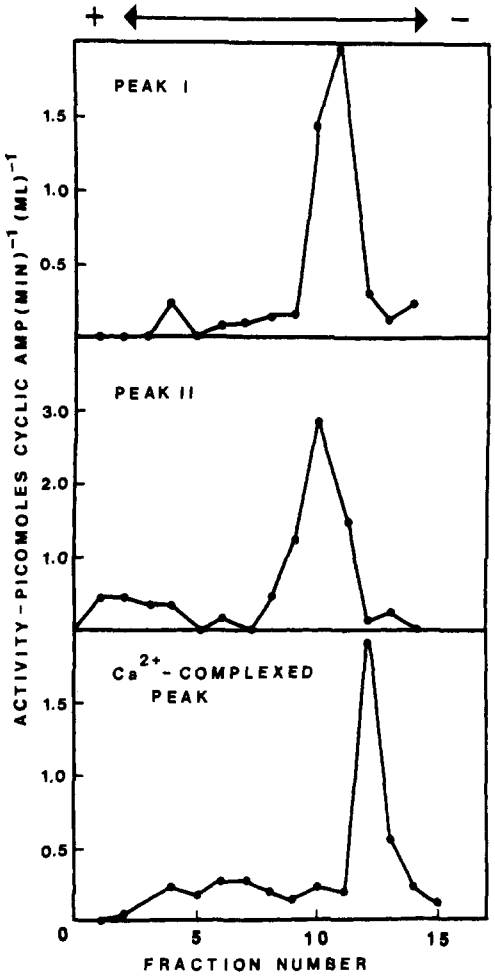

FIGURE 3: Analytical polyacrylamide gel electrophoresis of bovine heart phosphodiesterases. A 200-μL aliquot of peak I (105 μg of protein) and Ca^{2+}-complexed enzyme (30.2 μg of protein) isolated on the STMP-agarose column was applied to each gel as outlined under Materials and Methods. The approximate recovery of enzyme after extraction from the separating gel was: peak I, 15%; peak II, 42%; and Ca^{2+}-complexed enzyme, 41%. Electrophoretic migration is toward the left in the anodic direction.

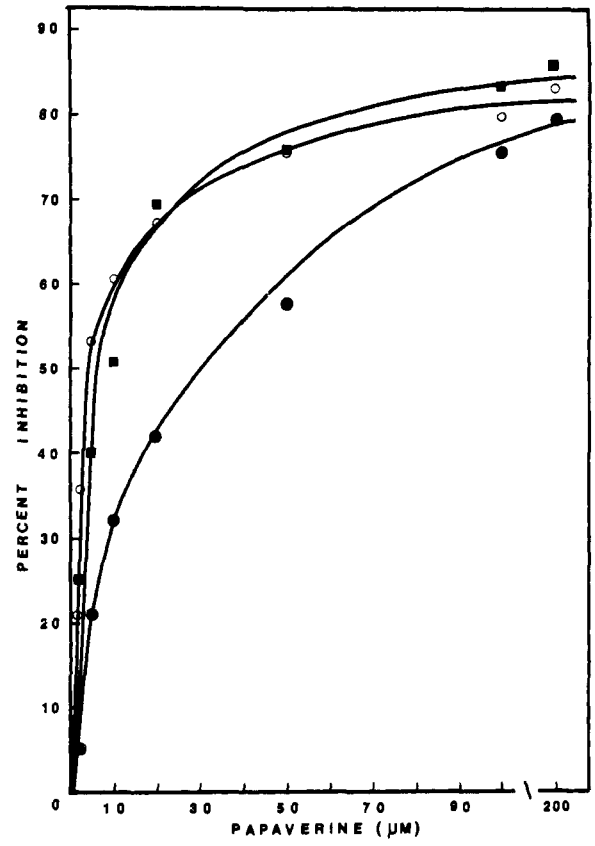

FIGURE 4: Inhibition of bovine heart phosphodiesterase by papaverine. Phosphodiesterases of peaks I (●) and II (○) isolated on the STMP-agarose column, and the ammonium sulfate fraction prepared from beef heart (■) were assayed for activity in the presence of increasing amounts of papaverine. The radiometric assay (procedure II) was used at 1 μM cyclic AMP.

not stimulated by trypsin treatment. Other experiments with snake venom pretreatment and the addition of saturating amounts of brain protein activator demonstrated that the activator-deficient enzyme was maximally stimulated to the same degree by either treatment.

Kinetic studies were performed to further characterize the two forms of phosphodiesterase isolated from heart. The results are shown in Table I. Apparent kinetic constants for the single peak of enzyme activity isolated in the presence of calcium (Ca^{2+}-complexed enzyme) are included for comparison. All enzymes showed normal Michaelis-Menten kinetics. The activator-responsive peak I was more specific for cyclic GMP than cyclic AMP based on the kinetic constants determined in either the presence or absence of protein activator. In the presence of the protein activator, the K_M of peak I for either nucleotide was only slightly reduced, while the maximum velocity increased 2.3-fold. The kinetic constants of peak II were approximately the same for both substrates.

The three forms of ventricular phosphodiesterase reproducibly displayed different electrophoretic mobilities relative to the tracking dye on polyacrylamide gel electrophoresis (Figure 3). The Ca^{2+}-complexed enzyme had the lowest mobility (R_f 0.26) and consistently yielded one band of activity in 5% acrylamide gels unsupplemented with calcium ion. The R_f values of peaks I and II were 0.29 and 0.36, respectively. On sucrose density gradients (5–20%) only peak I reproducibly traveled as one discrete form of enzyme activity (data not shown). The molecular weight of peak I as determined by the sucrose density gradient centrifugation (Martin and Ames, 1961) was 88 000.

The sensitivity to inhibition by papaverine of enzymes from peak I, peak II, and the 55% ammonium sulfate fraction applied to the STMP-agarose column was investigated (Figure 4). Peak I was less susceptible to inhibition by papaverine than either peak II or the ammonium sulfate fraction. The papaverine concentrations necessary for 50% inhibition (I_{50}) of peak I, peak II, and the heart enzyme before chromatography were 26.1, 4.22, and 8.91 μM, respectively. The difference in the papaverine I_{50} values of peaks I and II may not account entirely for their order of elution from STMP-agarose. Using isomeric succinylated trimethylpapaveroline, the I_{50} values were 294, 277, and 152 μM for peak I, peak II, and the ammonium sulfate fraction, respectively.

Differences in the sensitivities of peak I, peak II, and the calcium-complexed enzyme to inactivation by alkylating reagents were also observed. Peak II was more susceptible than the other enzymes to inactivation by *p*-chloromercuribenzoate. Two minutes of exposure to 4 mM *p*-chloromercuribenzoate produced a 60–65% inactivation of peak II. Peak I and the enzyme isolated in the presence of calcium exhibited only moderate inactivation (14–24%) with this reagent under the same conditions.

When the enzyme from heart was chromatographed on columns of 1-methyl-3-isobutyl-7-xanthineacetic acid sub-

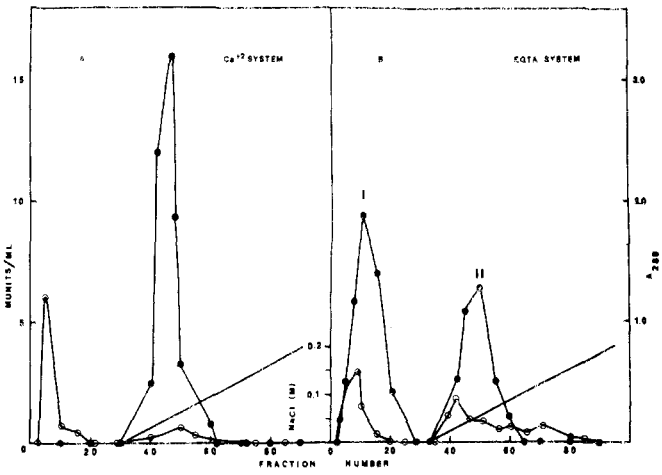

FIGURE 5: Chromatography of bovine heart phosphodiesterase on MIX-agarose. Protein (26.0 mg) was applied on a 1.6 × 3 cm column. Elution buffers contained either 100 μM $CaCl_2$ (panel A) or 100 μM EGTA (panel B). The column was washed with 100 mL of buffer A followed by a 200-mL gradient of NaCl in the same buffer. Other conditions are as described under Materials and Methods. Enzyme activity (●) was measured spectrophotometrically (procedure III) at 100 μM cyclic AMP. Absorbance (○) of each fraction was measured at 280 nm.

stituted agarose (MIX-agarose) in the presence of calcium or EGTA (Figure 5), elution profiles (measured at 100 μM cyclic AMP) under both conditions were similar to those observed on STMP-agarose. The enzymes, however, were eluted at lower ionic strengths when compared to the STMP-agarose columns. A single activity peak was obtained in the presence of calcium. The pooled fractions from this peak resulted in a fourfold purification but a yield of only 20%. In the presence of EGTA (Figure 5, right-hand panel), peak I was not retarded by the column and eluted in the wash before the gradient. Peak I was again activator responsive, being activated 4.8-fold by the addition of saturating amounts of protein activator from brain. Peak II was not activated under similar conditions.

Chromatography of the ammonium sulfate fraction from bovine brain cortex on MIX-agarose in the presence or absence of calcium is depicted in Figure 6A. In the presence of calcium, phosphodiesterase routinely emerged as three activity peaks: peak I eluted in the wash with unadsorbed proteins; peak II, the predominant activity, at the early stage of the gradient; and peak III during the final washing procedure with 150 mM NaCl (not shown in Figure 6A, Ca^{2+} system). Pooled fractions from peak II gave a yield of 39% and low purification based on the total activity applied to the column. Of the three enzyme forms eluted in the presence of calcium, only peak II was inhibited by EGTA.

With EGTA present, peak II was separated into two component peaks, IIa and IIb, as shown in panel A of Figure 6, EGTA system. Only peak IIA was stimulated by the protein activator from brain. The enzyme from peak IIA was maximally activated (2.8-fold) by the protein activator and showed a time-dependent activation in the presence of *Crotalus atrox* venom, similar to that observed for the activator-dependent peak I of bovine heart.

Chromatography of phosphodiesterase from brain cortex on STMP-agarose in the presence or absence of calcium is illustrated in Figure 6B. The elution profile of the enzyme in the presence or absence of calcium is similar to that observed on the MIX-agarose column, except that the resolution between peaks IIA and IIB in the EGTA system is not complete. Although not shown in Figure 6B, good correlation existed between the position of the peaks using either cyclic AMP or cyclic GMP at 0.1 μM. In the calcium system, peak I was not retarded by the column; peak II was eluted at 170 mM NaCl, and peak III in the final wash with 1 M NaCl. In the Ca^{2+} system, only peak II was inhibited by EGTA to the extent of 79%. No correlation was observed between the order of elution of the enzymes and their susceptibility to inhibition by papaverine. The I_{50} values for peaks I, II, and III isolated in the presence of calcium were 8.23, 14.1, and 10.9 μM, respectively. Of the peaks isolated on STMP-agarose in the presence of EGTA, only peaks IIA (4.6-fold) and IIB (1.9-fold) were stimulated by protein activator. The endogenous protein activator eluted between these two peaks. The small shoulder of the protein activator peak may be due to incomplete separation of peaks IIa and IIB.

Discussion

The behavior of phosphodiesterase on immobilized inhibitor columns is in agreement with recent observations of Ho et al. (1976) who used DEAE-cellulose and EGTA-supplemented elution buffer to separate two forms of phosphodiesterase from the soluble fraction of bovine heart. An analogous behavior was observed in the presence and absence of calcium. In the presence of EGTA, the activator-responsive enzyme emerged before the activator-unresponsive phosphodiesterase as observed on DEAE-cellulose (Ho et al., 1976). On inhibitor-substituted columns, the protein activator elutes at a position between the newly separated peaks. In contrast, the acidic activator elutes on DEAE-cellulose only at high ionic strength, well beyond any phosphodiesterase activity (Cheung, 1971).

A direct comparison between the properties of the enzymatic forms isolated from heart in this study and those isolated by earlier investigators (Goren and Rosen, 1972; Hrapchak and Rasmussen, 1972) appears difficult because of reasons discussed by Ho et al. (1976). However, the forms of phosphodiesterase isolated by this technique are comparable to the enzymes recently investigated by Ho et al. (1976) and by Donnelly (1977) who characterized the activator-dependent form of beef heart phosphodiesterase. In agreement with these studies, peak I activity is activator responsive and peak II is activator unresponsive; peak I is more cyclic GMP specific, whereas peak II has kinetic parameters indicative of a nonspecific cyclic nucleotide phosphodiesterase; the apparent Michaelis constants for the activator-responsive form of phosphodiesterase agree well with values reported by Donnelly (1977). In contrast to the normal Michaelis-Menten kinetics observed in this investigation, Ho et al. (1976) found nonlinear double-reciprocal kinetic plots for the activator-unresponsive form of the enzyme. Donnelly (1977) reported that the activator-responsive form of the enzyme gave nonlinear double-reciprocal plots which could be transformed to linear plots by the addition of the protein activator to the reaction mixtures. The disparity between these kinetic studies emphasizes the critical nature of the procedures used to isolate phosphodiesterase.

Ho et al. (1977) have suggested that the activator-unresponsive enzyme is a desensitized form of the activatable phosphodiesterase. This desensitization could be brought about by a proteolytic process; further, it was suggested that the interaction of activator-responsive phosphodiesterase with its protein activator in the presence of calcium causes the enzyme-activator complex to coelute with the activator-unresponsive enzyme. A similar behavior as observed on the immobilized inhibitor columns may indicate that the coelution of the two enzymes in the presence of calcium may not be coincidental. Under isolation procedures not employing EGTA,

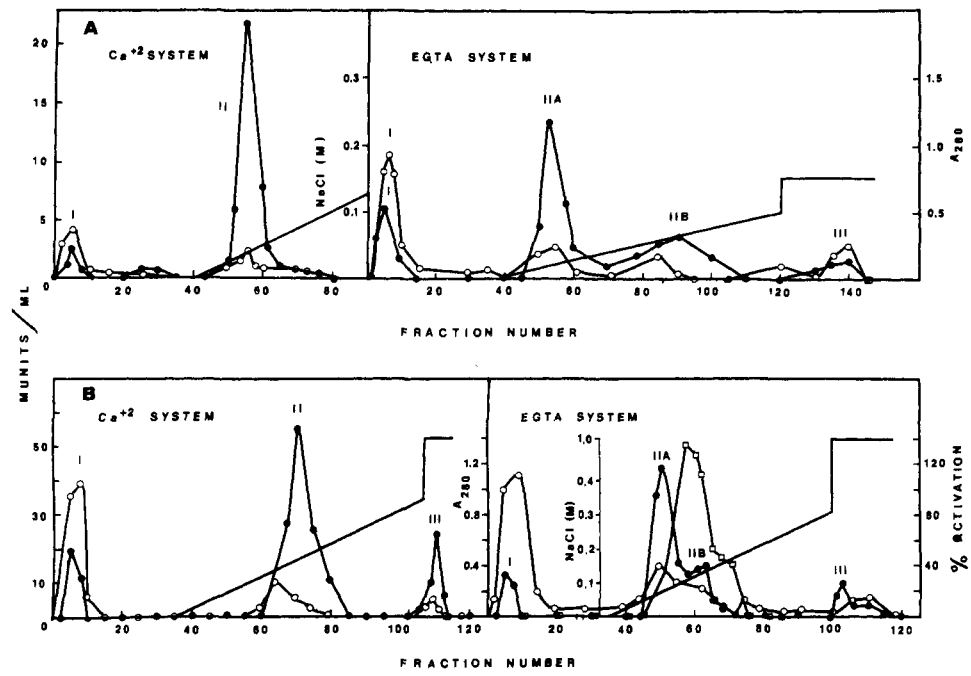

FIGURE 6: Chromatography of bovine brain cortex phosphodiesterase on MIX-agarose and STMP-agarose. Panel A: The 50% ammonium sulfate fraction of the 12 000*g* supernatant of bovine brain cortex (7.06 mg of protein, calcium system; 14.1 mg of protein, EGTA system) was applied on a 1.6 × 3 cm column of MIX-agarose. The column was washed with 100 mL of buffer A followed by a 200-mL linear gradient of NaCl in the same buffer. The final wash (EGTA system) was with 0.15 M NaCl in buffer A. Panel B: The 50% ammonium sulfate fraction of bovine brain cortex (35.2 mg of protein) was applied on a 1.6 × 6 cm column of STMP-agarose. The column was washed with 100 mL of buffer A followed by a 250-mL linear gradient of NaCl in the same buffer. The final wash was with 1.0 M NaCl in buffer A. Activator activity (□) is expressed as percent stimulation of peak IIA (fraction no. 48) by 250 μL of the heat-denatured fraction. Both columns were eluted with either 100 μM $CaCl_2$ (left-hand panels) or 100 μM EGTA (right-hand panels). Enzyme activity (●) was determined spectrophotometrically at 100 μM cyclic AMP (procedure III). Absorbance (○) of each fraction was measured at 280 nm. Other conditions are as described under Materials and Methods.

we have observed a constant degree of inhibition by EGTA at different stages of purification of the heart enzyme. This constancy of EGTA inhibition does not suggest a loss of Ca^{2+}-dependent activator or conversion to an activator-unresponsive form. Thus, the presence of an activator-unresponsive enzyme in these preparations does not appear to be due to an artifact introduced during the isolation procedure. The single peak of activity observed for the Ca^{2+}-complexed enzyme on polyacrylamide gel electrophoresis would also indicate a stable interaction between the two heart enzymes. The existence of a stable complex of two distinct phosphodiesterases may explain why purified enzyme preparations from different tissues still exhibit biphasic double-reciprocal kinetic plots (Thompson and Appleman, 1971a; Beavo et al., 1970). Although not observed in this study, the Ca^{2+}-complexed enzyme may give rise to biphasic kinetic patterns under certain conditions. The similar behavior of one form of phosphodiesterase from bovine brain cortex on the inhibitor-substituted columns gives added support to the possibility of a multienzyme–activator complex existing in the presence of calcium. Further investigations, however, will be required to firmly establish the origin of this second form of phosphodiesterase which appears in the presence of EGTA.

Attempts were made to investigate the biospecificity of the inhibitor-substituted columns. The low recovery and purification remain unexplained but may be partly due to the inability to utilize competitive-ligand elution and to interference of the inhibitor–substrate interaction by the support. That only one phosphodiesterase from columns possessing good resolution responds to protein activator or EGTA would suggest that selective separation between individual forms of phosphodiesterase occurred. Although the enzymes from brain cortex have not been characterized beyond their response to proteolytic treatment, EGTA, and protein activator, only one of the enzymes was responsive. Pledger et al. (1975) using polyacrylamide gel electrophoresis reported that four forms of phosphodiesterase occur in rat cerebrum after dialysis against EGTA. These same investigators also observed that only one enzyme was predominately responsive to activator. In supplemental studies, the brain preparation was chromatographed on agarose beads substituted only with the diaminodipropylamine extension. Although four peaks of phosphodiesterase activity were observed in the presence of EGTA, the major peaks, IIA and IIB (as designated in Figure 6), were diffuse and not resolved. Since extended hydrocarbon chains are capable of binding a number of enzymes through hydrophobic interaction (Shaltiel and Er-el, 1973), these results would suggest that separation is based predominately on hydrophobic interaction with some contributing biospecific interaction with the immobilized inhibitor. The latter force may be responsible for the stronger binding and differences in resolution between STMP-agarose and MIX-agarose. The similarity of the elution patterns between gels containing different ligands would also suggest that salt-sensitive hydrophobic interaction is the predominant force contributing to the separation process.

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Kinetics of *rac*-1-Oleyl-2-[4-(3-pyrenyl)butanoyl]glycerol Transfer between High Density Lipoproteins†

Steven C. Charlton, Kong-Yi Hong, and Louis C. Smith*,‡

ABSTRACT: The mechanism of transfer of diglyceride between high density lipoproteins (HDL) was investigated with a pyrene-containing analogue whose fluorescent properties depend on the microscopic concentration in the lipoprotein. Transfer rates were first order, rapid (3.5 s^{-1}), and invariant over a 100-fold range of HDL concentration and over a 10-fold range of *rac*-1-oleyl-2-[4-(3-pyrenyl)butanoyl]glycerol concentrations. Similar behavior of the probe was observed with HDL_3, although the rate was 40% slower. These results support a mechanism in which rate-limiting dissociation of the diglyceride analogue from one HDL particle into the aqueous phase precedes rapid diffusion and subsequent uptake by another such particle.

The equilibration of the phospholipids (Illingworth & Portman, 1972a,b), triglycerides (Quarfordt et al., 1971), and cholesterol (Bruckdorfer & Green, 1967; Basford et al., 1971) among various lipoproteins and tissue pools is a well-documented process. However, the time scale and the mechanism by which lipid transfer occurs in the absence of specific exchange proteins (Butler & Thompson, 1975; Wirtz et al., 1972) are not well established. Previous studies had poor time resolution and were complicated by the need to separate products of exchange. To circumvent these problems, we have used a probe containing pyrene, a molecule with fluorescence properties which depend on the local concentration of the compound.

Dilute solutions of pyrene exhibit a fluorescence from the lowest excited singlet state with a maximum at 390 nm. This is termed monomer fluorescence. At higher pyrene concen-

† From the Division of Atherosclerosis and Lipoprotein Research, Departments of Medicine and Biochemistry, Baylor College of Medicine and The Methodist Hospital, Houston, Texas 77030. *Received September 12, 1977; revised manuscript received May 4, 1978.* Support for the research was provided by grants from the Robert A. Welch Foundation Q-343, The American Heart Association 73-778, U.S. Public Health Service HL-15648 and HL-17269, and NIHLB Lipid Research Clinic Contract 71-2156.

‡ Established Investigator of the American Heart Association. Address correspondence to this author at: Baylor College of Medicine, Methodist Mail Station—A601, Houston, Texas 77030.

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