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CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITIES FROM PIG CORONARY ARTERIES

LACK OF INTERCONVERTIBILITY OF MAJOR FORMS *

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Summary

DEAE-cellulose chromatography, with or without dithiothreitol and over a pH range of 6.0 to 8.5, resolved two phosphodiesterase activities (peaks I and II) from the soluble fraction of pig coronary arteries. The activity of peak I was increased by calmodulin (3—7-fold), whereas that of peak II was not. Chromatography of peak I on Bio-Gel A-0.5 m columns resolved two peaks of phosphodiesterase activity (peaks Ia and Ib). Peak Ia was eluted in the presence or absence of 0.1 M KCl and was relatively insensitive to calmodulin. Peak Ib was eluted only in the presence of KCl and was sensitive to calmodulin. The substrate specificity and kinetic behavior were the same for peaks I, Ia, and Ib. Repeated gel chromatography of either peak Ia or Ib, under appropriate conditions, yielded a mixture of peaks Ia and Ib. Peak Ia appears to be a reversible aggregate of peak Ib. Gel chromatography of peak II resolved only one phosphodiesterase activity, which was eluted without KCl, was highly specific for cyclic AMP, was not sensitive to calmodulin and migrated differently on the gel column than either peak Ia or Ib.

Sucrose density gradient centrifugation of the soluble fraction from pig coronary arteries in the presence or absence of dithiothreitol resolved two peaks of phosphodiesterase activity (6.6 S and 3.6 S) which were similar to peaks I and II separated by DEAE-cellulose chromatography with regard to their substrate specificity and their sensitivity to calmodulin. Upon recentrifu-

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

gation, each of the two peaks of phosphodiesterase activity gave a single peak of activity which migrated with the same S value as did its parent.

These results indicate that the two major forms of phosphodiesterase of pig coronary arteries, which are representative of those found in many tissues, are not interconvertible in cell-free systems.

Introduction

Cyclic nucleotide phosphodiesterases play important roles in controlling cyclic nucleotide levels in mammalian cells [1,2]. Cell-free systems of many tissues and cell types contain separable forms of cyclic nucleotide phosphodiesterase which differ in their substrate specificity, kinetic properties, responsiveness to a Ca^{2+}-dependent regulator protein now commonly referred to as calmodulin [3], and sensitivity to other activators and inhibitors (see Ref. 2). The existence of all of the reported forms in intact cells is uncertain, however, since it has been reported that properties of some of the forms of cyclic nucleotide phosphodiesterase can be altered by proteolysis or by agents and/or conditions that affect sulfhydryl groups, net charge, or hydrophobic environment of the enzymes [4—7]. It has been suggested that some forms of phosphodiesterase are interconvertible in cell-free systems [7—10], and Epstein et al. have concluded that a single form of the enzyme from rat uterus can be converted by trypsin treatment into two forms with different kinetic properties and substrate specificities [7].

The general implication emerging from these reports is that the number and properties of phosphodiesterases that can be detected in cell-free systems are highly dependent on the techniques and conditions used for separation of the multiple forms. More specifically, the possibility is raised that two major forms of the enzyme that have been demonstrated in extracts from many tissues, often referred to as the 'high K_m' and the 'low K_m' forms, are interconvertible in cell-free systems. The soluble fraction of pig coronary arteries contains two forms of phosphodiesterase that can be separated by ion exchange chromatography [11]. One of these is activated by calmodulin, has a lower K_m for cyclic GMP than for cyclic AMP and generally displays properties of the so-called 'high K_m' form of phosphodiesterase found in many tissues. The other is insensitive to calmodulin, has a much greater specificity for cyclic AMP than for cyclic GMP and generally displays properties of the so-called 'low K_m' form. The studies to be reported here were undertaken to explore the possibility that the appearance of these two forms of phosphodiesterase is dependent upon conditions and techniques used for their separation and that these two forms are interconvertible in cell-free systems. We found no evidence to suggest that interconversion of the two forms occurs after homogenization or that various methods for their separation have a detectable effect on their properties.

Methods

Materials. Cyclic AMP and cyclic GMP (Sigma) were prepared as stock solutions and used without further purification. Tritiated cyclic nucleotides

obtained from New England Nuclear Corp. were purified on Dowex-50 cation exchange resin columns [12]. Ultrapure sucrose (density gradient grade) was obtained from Schwarz/Mann, benzamidine and DEAE-cellulose (0.95 mequiv./mg coarse grade) from Sigma, and Bio-Gel A-0.5 m (100—200 mesh) from Bio-Rad. Leupeptin was obtained from the U.S.-Japan Cooperative Research Program.

Soluble phosphodiesterase preparation. The media plus intima layers of pig coronary arteries were obtained as described previously [11], blotted on filter paper, weighed, minced with scissors, and homogenized in 4 ml/g (wet weight) of a solution containing 20 mM Tris-HCl (pH 7.5), 2 mM $MgOAc_2$ and 1 mM dithiothreitol at 4°C with an Ultra-Turrax homogenizer. The homogenate was centrifuged for 30 min at 48 000 × *g* at 4°C, and small aliquots of the supernatant fraction were stored at —70°C.

DEAE-cellulose chromatography. DEAE-cellulose chromatography of the soluble fraction and the preparation of peak I and II were conducted as described previously [11]. Peak fractions were pooled, concentrated to approx. 10% of the original volume in an Amicon ultrafiltration cell with a PM-10 membrane and dialyzed for 24 h against 100 vols. (3 changes) of 20 mM Tris-HCl (pH 7.5) containing 2 mM $MgOAc_2$ and 1 mM dithiothreitol (when indicated). These preparations were then stored at —70°C in small aliquots.

Gel chromatography. Bio-Gel A-0.5 m was packed into a 1.5 × 45 cm column and equilibrated with 20 mM Tris-HCl (pH 7.5), 2 mM $MgOAc_2$, 1 mM dithiothreitol and 100 mM KCl (when indicated). The enzyme preparation (2.5 ml in the appropriate buffer) was layered onto the gel, and the column was developed with the same buffer as used for equilibration. The flow rate was 8.6 ml/h, and 1.5-ml fractions were collected in tubes containing 2 mg of bovine serum albumin. The void volume was determined with Blue Dextran 2000, and the column was calibrated with bovine serum albumin, yeast alcohol dehydrogenase and catalase.

Sucrose density gradient centrifugation. The enzyme preparation (0.1 ml) was layered upon 5 ml of a sucrose gradient prepared with a Buchler gradient maker in 20 mM Tris-HCl (pH 7.5) containing 2 mM $MgOAc_2$ (when indicated), 1 mM dithiothreitol (when indicated), and 0.1 mM EGTA (when indicated). The sucrose concentrations were found by refraction measurements to consist of two successive linear gradients: the first of 4 ml (5 to 19% sucrose) and the second of 1.25 ml (20 to 32% sucrose). Peaks of enzyme activity sedimented within the first 4-ml gradient. Gradients were centrifuged in a Beckman SW 50L rotor for 15 h at 45 000 rev./min at 4°C. Fractions of 0.25 ml were collected manually from the top of the gradient. Gradients were calibrated to get approximate molecular weights with the following markers, cytochrome *c*, chymotrypsinogen *a*, hen egg albumin, bovine serum albumin, rabbit muscle aldolase and beef liver catalase. Sedimentation coefficients were calculated by the method of Martin and Ames [13]. Sucrose concentrations were determined with a Bausch and Lomb 377SD refractometer.

Phosphodiesterase assay. The assay of phosphodiesterase activity was as described previously [11] with the following modification. The medium contained 48 mM Tris-HCl (pH 7.5), 2 mM $MgOAc_2$, 10 μM $CaCl_2$, 1 mg/ml of bovine serum albumin (Fraction V, Sigma), a saturating amount of purified cal-

modulin (when indicated) and/or 1 mM EGTA plus 1 mM $MgOAc_2$ (when indicated). The reaction was started by addition of the phosphodiesterase preparation or the substrate and stopped by the addition of 25 μl of an aqueous solution of 50 mM EDTA, 30 mM theophylline, 10 mM cyclic AMP, 10 mM cyclic GMP and 100 mM Tris-HCl (pH 7.5). Excess *Crotalux atrox* venom (0.2 mg) was then added in a volume of 10 μl, and the solution was incubated for 20 min at 30°C. After incubations were completed, the reaction mixture was diluted to 1 ml with an aqueous solution of 0.1 mM adenosine, 0.1 mM guanosine, and 15 mM EDTA. The tritiated, dephosphorylated products were separated from tritiated nucleotides by small QAE-Sephadex formate columns [11], and the tritiated dephosphorylated products were counted in 10 ml of ACS scintillation fluid (Amersham).

Product accumulation was determined to be linear with time and with enzyme dilution under all conditions studied.

Pig brain calmodulin preparation. Calmodulin was purified from pig brain to near homogeneity by the procedure of Lin et al. [14], except that the last step was hydroxylapatite chromatography instead of preparative acrylamide gel electrophoresis. Hydroxylapatite was packed into a 0.9×58 cm column, equilibrated and eluted with 25 mM PO_4^{3-} (pH 6.8) and 10 μM Ca^{2+}. The active fractions were pooled, concentrated through a Diaflo PM-10 membrane and then stored at −70°C in small aliquots. In this paper 'stimulation' of phosphodiesterase activity by calmodulin means the increase in activity seen when saturating concentrations (more than 2.4 μg/ml) of calmodulin and 10 μM Ca^{2+} were added to an assay of calmodulin-deficient enzyme.

Protein was determined as described by Lowry et al. [15], using bovine serum albumin as standard.

Results

DEAE-cellulose chromatography

The 48 000 × *g* supernatant fraction from pig coronary arteries was resolved into two phosphodiesterase activities (peak I and II) and a Ca^{2+}-dependent activator protein by DEAE-cellulose chromatography at pH 7.5 in the presence of dithiothreitol, as described previously [11]. Peak I catalyzed the hydrolysis of both cyclic AMP and cyclic GMP and had a lower K_m for cyclic GMP than for cyclic AMP. Peak II was highly selective for cyclic AMP. Peak I was stimulated by calmodulin (3—6-fold) whereas peak II was not. When chromatography was performed at pH 8.5 in the presence of 1 mM dithiothreitol, the 48 000 × *g* supernatant fraction was similarly resolved in two phosphodiesterase activities (Fig. 1). There was no significant modification in the pattern of the separation when chromatography was performed at pH 6.0 or at pH 7.5 in the absence of presence of 1 mM dithiothreitol or in the presence of 1 mM benzamidine or 5 μM leupeptin.

In the work reported in this paper, all preparations of peaks I and II were derived from the DEAE-cellulose chromatography of fresh supernatant fractions from coronary arteries. In one experiment, however, an aliquot of a fresh supernatant fraction was applied to DEAE-cellulose and another aliquot of the same supernatant fraction was allowed to incubate for 10 days at 4°C before

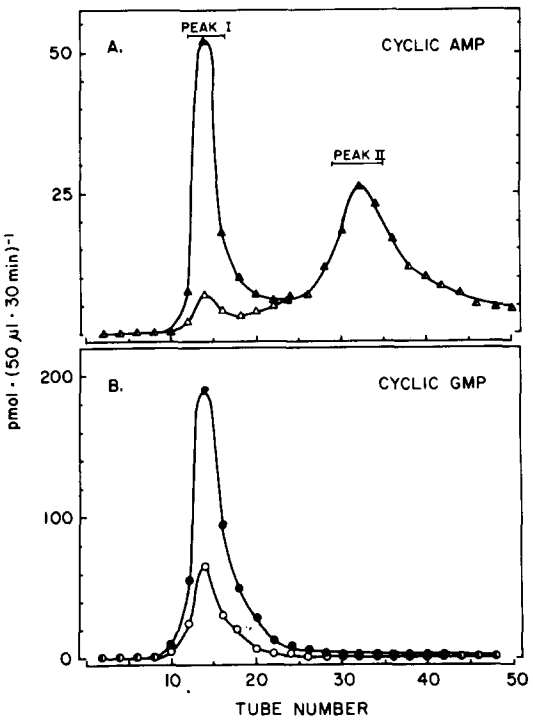

Fig. 1. DEAE-cellulose chromatography of the 48 000 × *g* supernatant fraction from pig coronary arteries. The supernatant fraction prepared as described in Methods (2.6 ml) was adjusted to pH 8.5 with 20 mM Tris-HCl and then applied to a 10 × 0.9 cm column of DEAE-cellulose previously equilibrated with 20 mM Tris-HCl (pH 8.5) containing 2 mM $MgOAc_2$ and 1 mM dithiothreitol. The column was developed with the same buffer containing an exponential gradient of $(NH_4)_2SO_4$, as described by Wells et al. [11]. The flow rate was 0.6 ml/min, and 8-ml fractions were collected in tubes containing 8 mg bovine serum albumin in 0.2 ml H_2O. Cyclic GMP (○,●) and cyclic AMP (△,▲) phosphodiesterase activities were assayed in the presence (●, ▲) or absence (○, △) of calmodulin. Based on the phosphodiesterase activity assayed in presence of calmodulin, the recoveries were estimated to be 85% for cyclic AMP and 63% for cyclic GMP.

being subjected to DEAE-cellulose chromatography (data not shown). The elution profiles of the two fractionations were indistinguishable with respect to elution volume and substrate specificity when assayed with either 1 μM cyclic AMP or 1 μM cyclic GMP. However, peak I from the aged supernatant fraction was stimulated only 27% by the addition of calmodulin, whereas the activity of peak I from the fresh supernatant fraction was stimulated 4-fold. On the other hand, total activity (assayed with saturating calmodulin) recovered under peak I was nearly identical in the two elution profiles. Thus, the only detected change in the phosphodiesterase activities caused by incubation for 10 days at 4°C was an increase in the basal (no added calmodulin) activity of peak I. The same results were obtained when pooled and dialyzed peak I from DEAE-cellulose chromatography was aged for 10 days and then rechromatographed on DEAE-cellulose.

Gel chromatography of peaks I and II

Chromatography of peak I on Bio-Gel A-0.5 m columns in buffer containing

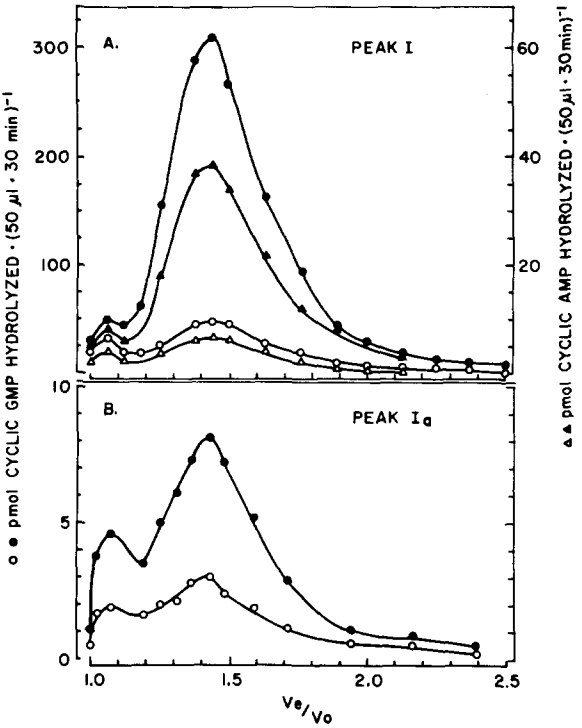

Fig. 2. Bio-Gel A-0.5 m chromatography of peak I (A) and rechromatography of peak Ia (B). The column was equilibrated with Tris-HCl buffer containing 100 mM KCl as described in Methods. The enzyme preparation (2.5 ml in 100 mM KCl) was layered onto the column, and the column was developed with the same buffer. The flow rate was 8.6 ml/h and 60 fractions of 1.5 ml each were collected in tubes containing 2 mg of bovine serum albumin in 0.05 ml of H_2O. Phosphodiesterase activities were assayed with 1 µM cyclic GMP (○, ●) and 1 µM cyclic AMP (△, ▲) in the presence (●, ▲) or absence (○, △) of calmodulin. The recoveries of added cyclic GMP phosphodiesterase activity in Panel A were estimated to be 120% and 80%, respectively, in the presence and the absence of calmodulin. The recoveries of added cyclic GMP phosphodiesterase activity in panel B were estimated to be 131% and 96%, respectively, in the presence and absence of calmodulin.

0.1 m KCl (Fig. 2A) resolved two peaks of phosphodiesterase activity. The activity of the first, smaller peak was increased only slightly by calmodulin, but that activity of the second peak was increased 5—8-fold by this protein (Fig. 2A). These two peaks hydrolyzed both cyclic AMP and cyclic GMP, and the ratio of cyclic GMP to cyclic AMP phosphodiesterase activity was the same for the two peaks and for peak I from the DEAE-cellulose column. The first peak eluted with V_e/V_0 (ratio of elution volume to void volume) value of 1.06 and the second with a value of 1.43.

In contrast to the results shown in Fig. 2A, when peak I was eluted from the gel column in the absence of KCl (Fig. 3A), only a small peak of activity (peak Ia) was recovered. The activity of this peak was only slightly stimulated by calmodulin (0—2-fold, depending on the experiment) but was not inhibited by EGTA. The major portion of the activity could be recovered from the column by subsequent elution with buffer containing 0.1 M KCl. The peak thus eluted (Ib) was highly activatable by calmodulin (4—9-fold, depending upon

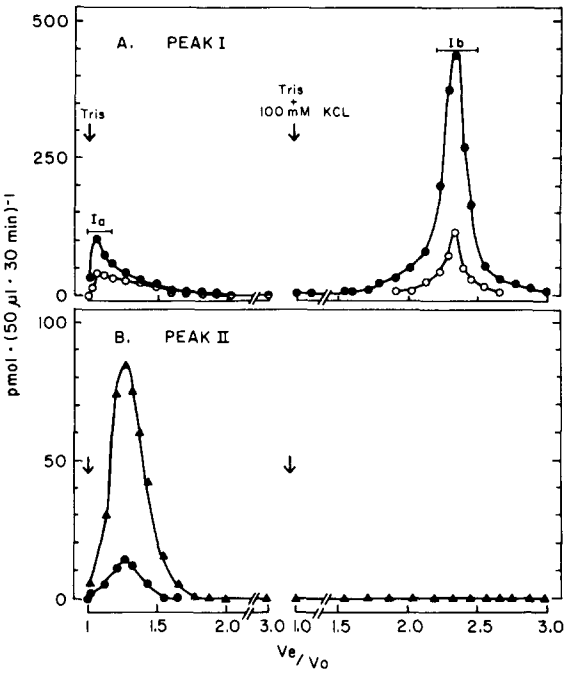

Fig. 3. Bio-Gel A-0.5 m gel chromatography of peak I (A) and peak II (B) from DEAE-cellulose chromatography. The column was equilibrated with Tris-HCl buffer, loaded with peak I or peak II (2.5 ml in Tris-HCl buffer), and developed with the same buffer. After collecting 60 fractions, the column was then developed with Tris-HCl buffer containing 100 mM KCl (see arrow) and 80 fractions were collected. The flow rate was 8.6 ml/h and 1.5-ml fractions were collected in tubes containing 2 mg of bovine serum albumin in 0.05 ml of H_2O. A, phosphodiesterase activity was assayed with 1 µM cyclic GMP in the presence (•) or the absence (○) of calmodulin. The recovery of cyclic GMP phosphodiesterase activity assayed with and without calmodulin was estimated to be 17% and 45%, respectively in the absence of KCl, and 103% and 65%, respectively, in the presence of KCl. B, Phosphodiesterase activities were indistinguishable in the absence or presence of calmodulin with 1 µM cyclic GMP (•) and with 1 µM cyclic AMP (▲). The cyclic AMP phosphodiesterase activity recovered was 104%.

the experiment). The cyclic GMP and cyclic AMP phosphodiesterase activities in peaks I, Ia, and Ib displayed rectilinear kinetic plots with an apparent K_m of 1.5—3.0 µM for cyclic GMP and 25—72 µM for cyclic AMP; the ratio of the V values for the two substrates were the same for peaks I, Ia, and Ib. Thus, the three peaks did not appreciably differ in their specificity or kinetic behavior.

When peak Ia was reapplied to the gel column in presence of 0.1 M KCl and eluted with buffer containing the salt (Fig. 2B), two peaks were resolved, corresponding in their elution volumes to the two peaks obtained when the original peak I was applied in the presence of KCl. When reapplied to the gel column in absence of KCl, peak Ib (previously dialyzed against Tris-HCl buffer to remove KCl) yielded a small amount of activity that eluted in the position of peak Ia and was not sensitive to calmodulin; the major part of peak Ib was retained by the column until 0.1 M KCl was added (data not shown). Peak Ia thus appeared to be a reversible aggregate of peak Ib that was relatively insensitive to calmodulin but retained the kinetic behavior and substrate specificity of peak Ib.

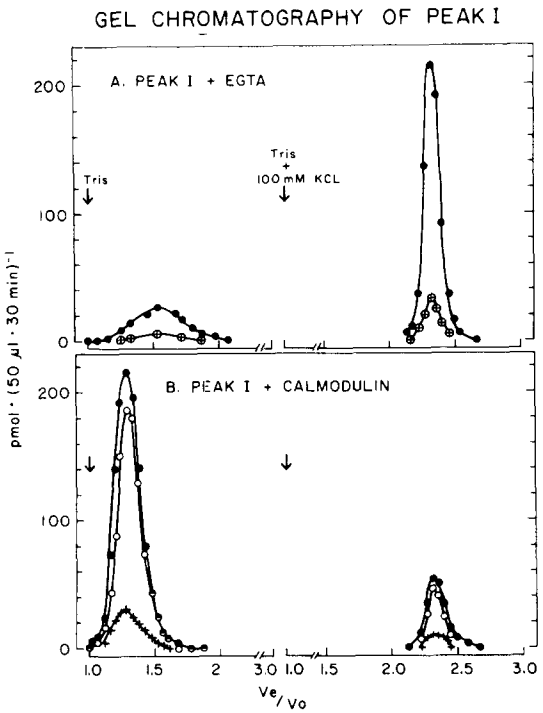

Fig. 4. Gel chromatography of peak I. (A) Peak I from DEAE-cellulose was made 1 mM with EGTA (final concentration). The buffers for equilibration of the column and elution were the same as those used in the experiments depicted in Fig. 3, except they contained 0.1 mM EGTA. (B) Peak I (2.5 ml in Tris-HCl containing 10 µM Ca^{2+}) was mixed with an excess of calmodulin (0.5 mg) before being loaded onto the column. The experimental conditions were the same as those described in the legend of Fig. 2. Phosphodiesterase activity was assayed in each fraction with 1 µM cyclic GMP in presence of calmodulin (•), in the absence of added calmodulin (○), and in the presence of 0.1 mM EGTA (+). In the experiment shown in panel A, 20% of the added phosphodiesterase activity was eluted without KCl and 89% was eluted with KCl. In panel B, 88% of the added activity was eluted in the absence of KCl and 15% was eluted in the presence of KCl.

Chromatography of peak II on Bio-Gel columns gave only one peak of phosphodiesterase activity (Fig. 3B), which was highly specific for cyclic AMP, neither activated by calmodulin nor inhibited by EGTA and entirely recovered in the absence of KCl. The V_e/V_0 value of peak II was distinctly different from those of peak Ia or Ib in the presence or absence of KCl.

When peak I was chromatographed without added calmodulin (Fig. 3A) or in the presence of EGTA (Fig. 4A), the major part of the activity was recovered only in presence of KCl, whereas when peak I was chromatographed in the presence of an excess of calmodulin and Ca^{2+} (Fig. 4B), the major part of phosphodiesterase activity was eluted without KCl in an activated state (not sensitive to the further addition of calmodulin and Ca^{2+} but inhibited by EGTA).

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation of the 48 000 × *g* supernatant fraction from pig coronary arteries (Fig. 5) resolved two phosphodiesterase activ-

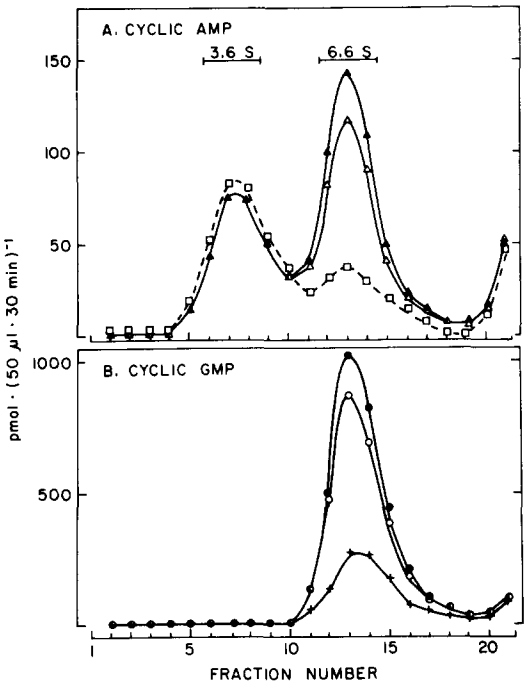

Fig. 5. Sucrose density gradient centrifugation of the 48 000 × *g* supernatant fraction from pig coronary arteries. The gradient and the enzyme loaded were prepared in 20 mM Tris-HCl (pH 7.5) containing 2 mM $MgOAc_2$ and 1 mM dithiothreitol. Fractions (0.25 ml) were collected in tubes containing 0.25 mg of bovine serum albumin in 0.01 ml of H_2O. Other experimental details are given in Methods. Phosphodiesterase activities were assayed with 1 µM cyclic GMP (●, ○, +) and 1 µM cyclic AMP (△, ▲, □) in presence (●, ▲) or absence (○, △) of added calmodulin, or in presence of 0.1 mM EGTA (+, □). Based on the phosphodiesterase activities assayed in the presence of calmodulin, the recoveries were 91% and 100%, respectively, with cyclic GMP and cyclic AMP.

ities with sedimentation coefficients of 3.6 S and 6.6 S. The 6.6 S peak catalyzed the hydrolysis of both cyclic AMP and cyclic GMP. The ratio of cyclic GMP to cyclic AMP phosphodiesterase activity was indistinguishable from the ratio in peak I from DEAE-cellulose at 1 µM substrate. The activity of this peak was only slightly increased by the addition of calmodulin, but it was markedly decreased by EGTA. the 3.6 S peak was relatively specific for cyclic AMP, and the activity was neither stimulated by calmodulin nor decreased by EGTA. The approximate molecular weights estimated from sucrose density gradient centrifugation of the standard proteins were about 45 000 for the 3.6 S peak and 140 000 for the 6.6 S peak.

The phosphodiesterase activity profiles obtained in the presence (Fig. 5) or absence of dithiothreitol and Mg^{2+} (Fig. 6A) were similar with regard to the number, sedimentation coefficients, substrate specificity and sensitivity to calmodulin of the peaks resolved. The profile shown in Fig. 6A was representative of profiles of three gradients run simultaneously. The phosphodiesterase activity of fraction 7 corresponded to that activity isolated as peak II from DEAE-cellulose chromatography. Fractions 7 from each of the three gradients were pooled and dialyzed against 20 mM Tris-HCl buffer, pH 7.5, in order to remove sucrose. The dialyzed, pooled fractions 7 were resubmitted to a sucrose

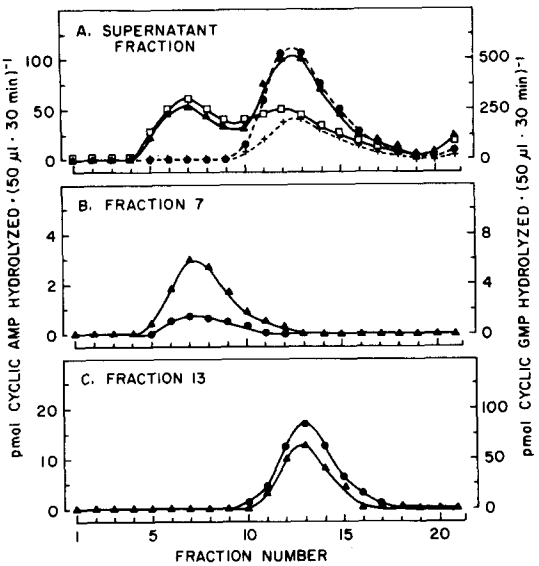

Fig. 6. Sucrose density gradient centrifugation of (A) pig coronary artery supernatant fraction, (B) pooled and dialyzed fractions 7 from Panel A and (C) pooled and dialyzed fractions 13 from Panel A. The gradients and the enzymes loaded were prepared in 20 mM Tris-HCl (pH 7.5). Other experimental details are given in Methods and in the text. Phosphodiesterase activities were assayed with 1 µM cyclic GMP (●, +) and 1 µM cyclic AMP (▲, □) in presence of calmodulin (●, ▲) or in presence of EGTA (+, □). Recoveries Recoveries of added phosphodiesterase activity were: A, 80% and 94% based on the activity assayed in presence of calmodulin for cyclic GMP and cyclic AMP, respectively; B, 64% for cyclic AMP; C, 100% for both cyclic AMP and cyclic GMP.

density gradient centrifugation and gave only one peak of activity which migrated the same as did its parent (Fig. 6B). Fractions 13 from each of the three gradients, when resubmitted to sucrose gradient centrifugation, likewise gave only one peak of phosphodiesterase activity which migrated virtually the same as did its parent (Fig. 6C). These experiments were repeated several times, in presence or absence of dithiothreitol and Mg^{2+}, and in presence or absence of bovine serum albumin in the collecting tubes. These different conditions did not affect the results.

Peak I separated from the 48 000 × *g* supernatant fraction by DEAE-cellulose was submitted to sucrose density gradient centrifugation (Fig. 7B). The resulting peak, depending upon the experiment, migrated as a 6.1 S form (Fig. 7B) or as a 5.6 S form (data not shown). Both the 6.1 S and the 5.6 S forms were stimulated by calmodulin, and they had approximate molecular weights of 124 000 and 108 000, respectively. When peak I from DEAE-cellulose was mixed with an excess of calmodulin and then submitted to a sucrose density gradient centrifugation, it migrated as a 6.6 S peak (Fig. 7C). Sucrose density gradient centrifugation of the 48 000 × *g* supernatant fraction in presence of 0.1 mM EGTA (to dissociate the endogenous calmodulin from the phosphodiesterase) resolved the phosphodiesterase activities into peaks 3.6 S and 5.6 S (data not shown). The differences between the S values of the peaks resulting

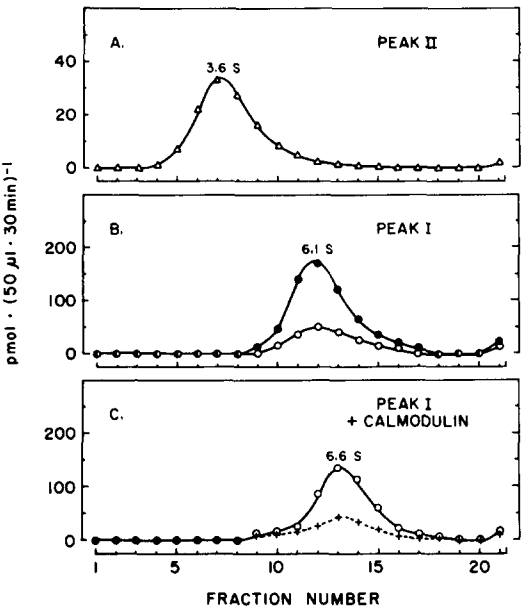

Fig. 7. Sucrose density gradient centrifugation of peak II, peak I, and peak I in the presence of calmodulin. Experimental conditions were the same as those described for Fig. 5 and in Methods. Panel A, Peak II from DEAE-cellulose chromatography of the soluble fraction from pig coronary arteries. Panel B, Peak I from DEAE-cellulose chromatography of the soluble fraction from pig coronary arteries. Panel C, Peak I was mixed with an excess of calmodulin before centrifugation. Phosphodiesterase activities were assayed with 1 µM cyclic AMP (Δ) or 1 µM cyclic GMP (●, ○, +) in the presence (●) or absence of calmodulin (○) or in the presence of 0.1 mM EGTA (+). Recoveries of added phosphodiesterase activity were: A, 92%; B, 107% and 117% for activity assayed in the absence and presence of calmodulin, respectively; C, 82% and 109% for activity assayed in the absence of calmodulin and in the presence of EGTA, respectively.

from peak I from DEAE-cellulose (Fig. 7B) and the 6.6 S peak sedimented from the supernatant fraction (Fig. 5) might have been due to one or two molecules of calmodulin being bound to the enzyme, but our present data do not permit a firm conclusion. Recent data from studies of cross-linking between purified enzyme and [^{125}I]calmodulin indicate that phosphodiesterase binds 2 mol of calmodulin per mol of enzyme [16]. It is clear, however, that the two phosphodiesterase forms separated from the crude supernatant fraction by density gradient centrifugation were indistinguishable from peaks I and II separated on DEAE-cellulose.

Epstein et al. have reported that what appears to be a single form of phosphodiesterase from the uterus of 20-day old rats is converted into two forms with substrate specificities that differ from each other and from the parent enzyme fraction when treated with trypsin [7]. Since peak I superficially resembles the properties reported for the rat uterine enzyme, at least in that both cyclic AMP and cyclic GMP are substrates for both these preparations, we treated peak I with trypsin under conditions (10 µg trypsin/ml of enzyme preparation incubated at 30°C for 5 min) that we had determined would increase the activity of this enzyme. Sucrose gradient centrifugation of peak I after treatment with trypsin revealed only one peak of phosphodiesterase

activity. This peak had the same ratio of cyclic AMP to cyclic GMP hydrolytic activity as did the fraction obtained by sucrose gradient centrifugation of the control (peak I that was preincubated in the absence of trypsin). However, the trypsin-treated fraction migrated with an apparent molecular weight of 95 000 and was insensitive to calmodulin, whereas the control preparation migrated with an apparent molecular weight of 124 000 and was sensitive to calmodulin.

Discussion

The data obtained from DEAE-cellulose chromatography, Bio-Gel chromatography and sucrose density gradient centrifugation experiments indicate that the two major forms of phosphodiesterase from pig coronary arteries are not interconvertible in cell-free systems. The phosphodiesterase activity of the 48 000 × *g* supernatant fraction from pig coronary arteries was resolved into two forms (peaks I and II) by DEAE-cellulose chromatography and by sucrose density gradient centrifugation. Neither pH, dithiothreitol, nor the protease inhibitors benzamidine and leupeptin affected the number of forms separated by these techniques, in contrast to the variation in the forms isolated from the rat kidney [5] under similar conditions. Peak II was relatively specific for cyclic AMP, was not sensitive to calmodulin, behaved as single form on gel chromatography and sucrose gradient centrifugation, and had a sedimentation coefficient of 3.6 S and an apparent molecular weight of 45 000 estimated by sucrose density gradient centrifugation. The apparent molecular weight of peak II as estimated from gel filtration with or without KCl (more than 100 000) was considerably greater than that estimated by sucrose density gradient centrifugation, which suggests a large degree of asymmetry within this molecule or its aggregation during gel filtration. Peak I hydrolyzed both cyclic AMP and cyclic GMP, was sensitive to calmodulin, had a sedimentation coefficient of 5.6 S and an apparent molecular weight of 108 000 when not associated with calmodulin. Peak I seemed to undergo some reversible aggregation during gel chromatography, but the aggregated and unaggregated forms of peak I appeared to differ only in their relative abilities to be activated by calmodulin. The major portion of peak I phosphodiesterase activity could be retained on gel columns, presumably due to ionic interactions with the gel matrix, in the presence of low ionic strength buffer and the absence of calmodulin. Others have noted that phosphodiesterase activities migrate with different apparent molecular weights during gel filtration in the presence or absence of salt [8,17].

The stimulation of phosphodiesterase activity by proteolytic enzymes has been known for several years [6,18—20]. Sakai et al. reported [6] that trypsin treatment of a crude phosphodiesterase preparation from rat brain increased the activity by generation of a new form with an apparent molecular weight of 80 000 accompanied by the loss of a form with an apparent molecular weight of 150 000. It is known [6,19] that the calmodulin-sensitive form of phosphodiesterase can be stimulated by trypsin treatment and that the resulting enzyme activity is no longer sensitive to calmodulin. We have demonstrated that the enzyme generated by trypsin treatment of the calmodulin-sensitive form of phosphodiesterase from coronary arteries is not the same form as peak II isolated from DEAE-cellulose. The peak II enzyme and peak I before or after

trypsin treatment were distinctly different in their migration on sucrose density centrifugation and in their substrate specificities. In addition, we have observed that storage at 4°C for several days of the supernatant fraction from a homogenate of coronary arteries leads to no change in the DEAE-cellulose profile of the phosphodiesterase activities. The peaks of activity (peaks I and II) eluted at identical salt concentrations had indistinguishable substrate specificities and had the same total activity as those from DEAE-cellulose chromatography of the fresh supernatant fraction; the only detectable alteration was that peak I had been rendered insensitive to calmodulin by the storage at 4°C. It is likely that this aging phenomenon represents the action of an endogenous protease on the calmodulin-sensitive form of phosphodiesterase.

Epstein et al. reported [7] that phosphodiesterase activity in extracts from uteri of 20-day-old rats appeared to behave as a single form of phosphodiesterase that catalyzed the hydrolysis of both cyclic AMP and cyclic GMP and that was converted by trypsin treatment into two forms with distinctly different substrate specificities. The data could be interpreted to indicate that a single enzyme could be converted into a mixture of peak I and peak II type phosphodiesterases. We have not been able to isolate a single enzyme by DEAE-cellulose chromatography of supernatant fraction from the uteri of 20-day-old Sprague-Dawley rats. The uteri were dissected and homogenized according to the methods utilized by Epstein et al. but were chromatographed on DEAE-cellulose by the methods reported here and elsewhere [11]. We obtained a calmodulin-sensitive fraction of activity and a second major fraction that corresponded in elution from DEAE-cellulose and in substrate specificity to peak II from coronary arteries. These same fractions were obtained whether or not the homogenization and chromatographic buffers contained 5 μM leupeptin and 10 mM sodium fluoride. We, therefore, must speculate that what appeared to be generation of two new forms by trypsin treatment of rat uterine phosphodiesterase [7] was the result of the use of techniques that were unable to resolve multiple forms present before trypsin treatment.

In work to be reported elsewhere, we have also been unable to reproduce the data of Pichard and Cheung [10] that was interpreted to suggest that different aggregated states of phosphodiesterase accounted for the multiple forms seen by sucrose gradient centrigugation of a crude preparation from rat liver. In our hands sucrose gradient centrifugation of the 48 000 $\times g$ supernatant fraction from fresh rat liver resolves two peaks of phosphodiesterase activity whether assayed at 100 μM or 1 μM substrates. Each of these peaks, when recentrifuged on a sucrose gradient, migrates to the same density as the parent peak with no other detectable fractions of activity. Also in contrast to the previous paper [10], we have been unable to detect any change in specific activity of the phosphodiesterase activity in crude extracts from fresh rat liver with changes in the amount of protein utilized in the assay or any changes in the kinetic behavior of this preparation when the protein concentration in the assay is varied. We are unable to account for the discrepancies between our results and those previously reported but must conclude that interconversion of separable 'high K_m' and 'low K_m' forms of phosphodiesterase remains to be convincingly and reproducibly demonstrated.

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