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Phosphorylation and Characterization of Bovine Heart Calmodulin-Dependent Phosphodiesterase†

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ABSTRACT: Calmodulin-dependent phosphodiesterase was purified to apparent homogeneity from the total calmodulin-binding fraction of bovine heart in a single step by immunoaffinity chromatography. The isolated enzyme had significantly higher affinity for calmodulin than the bovine brain 60-kDa phosphodiesterase isozyme. The cAMP-dependent protein kinase was found to catalyze the phosphorylation of the purified cardiac calmodulin-dependent phosphodiesterase with the incorporation of 1 mol of phosphate/mol of subunit. The phosphodiesterase phosphorylation rate was increased severalfold by histidine without affecting phosphate incorporation into the enzyme. Phosphorylation of phosphodiesterase lowered its affinity for calmodulin and Ca^{2+}. At constant saturating concentrations of calmodulin (650 nM), the phosphorylated calmodulin-dependent phosphodiesterase required a higher concentration of Ca^{2+} (20 μM) than the nonphosphorylated phosphodiesterase (0.8 μM) for 50% activity. Phosphorylation could be reversed by the calmodulin-dependent phosphatase (calcineurin), and dephosphorylation was accompanied by an increase in the affinity of phosphodiesterase for calmodulin.

Calmodulin-dependent cyclic nucleotide phosphodiesterase (3′,5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) is one of the key enzymes involved in the complex interactions between the cyclic nucleotide and Ca^{2+} second-messenger systems. Most tissues examined have been shown to contain calmodulin-dependent cyclic nucleotide phosphodiesterase activity [for reviews, see Beavo et al. (1982), Sharma et al. (1988), Beavo (1990), and Wang et al. (1990)]. The enzyme has been purified close to homogeneity from both bovine brain (Morrill et al., 1979; Sharma et al., 1980; Kincaid & Vaughan, 1983; Kincaid et al., 1984; Shenolikar et al., 1985) and bovine heart (LaPorte et al., 1979) and extensively characterized. Recent studies showed that calmodulin-dependent cyclic nucleotide phosphodiesterase exists in different isozymic forms (Hansen & Beavo, 1982; Sharma et al., 1984; Purvis et al., 1981; Vandermeers et al., 1983; Sharma & Wang, 1986a; Rossi et al., 1988) with different affinities for calmodulin (Mutus et al., 1985; Hansen & Beavo, 1986). Bovine brain calmodulin-dependent phosphodiesterase isozymes are differently regulated by phosphorylation (Sharma & Wang, 1985, 1986b,c). One of the isozyme forms, the 60-kDa subunit-containing isozyme from bovine brain, has been shown to be phosphorylated by adenosine cyclic 3′,5′-phosphate (cAMP)[1]-dependent protein kinase (Sharma & Wang, 1985, 1986b).

In this study, we examined the possible regulation of bovine heart calmodulin-dependent phosphodiesterase by cAMP-dependent protein kinase and observed that the rate of phosphorylation of this enzyme was significantly enhanced when histidine buffer was used in the phosphorylation reaction. Phosphorylation was accompanied by a decrease in affinity

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate.

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for calmodulin and Ca^{2+}, and could be reversed by dephosphorylation catalyzed by the calmodulin-dependent phosphatase (calcineurin).

Experimental Procedures

Materials

Bovine brain calmodulin was purified by phenyl-Sepharose column chromatography as described (Gopalakrishna & Anderson, 1982) and further purified by gel filtration on a Sephacryl S-200 column equilibrated with buffer A (20 mM Tris-HCl, 1 mM magnesium acetate, and 1 mM imidazole, pH 7.0) containing 0.1 M NaCl. Bovine brain calmodulin-dependent phosphatase (calcineurin) was purified as described previously (Sharma et al., 1983). Bovine heart cAMP-dependent protein kinase catalytic subunit was purified to homogeneity as described (Demaille et al., 1977). WIPTIDE, an inhibitor of the protein kinase, was purchased from Peninsula Laboratories. A monoclonal antibody, C1, which is specific for the 60-kDa phosphodiesterase from bovine brain, was produced and purified as described (Sharma et al., 1984). The antibody–Sepharose 4B conjugate used for affinity chromatographic purification of the calmodulin-dependent phosphodiesterase from bovine heart was prepared as described (Sharma et al., 1984). [γ-^{32}P]ATP was purchased from ICN.

Methods

Purification of Calmodulin-Dependent Phosphodiesterase. Early steps in the preparation of calmodulin-dependent phosphodiesterase from bovine heart, including the calmodulin–Sepharose 4B chromatographic stage, were as described previously (Sharma, 1990). Calmodulin–Sepharose 4B column chromatography was used to partially purify the calmodulin-dependent phosphodiesterase. However, the presence of other calmodulin-binding proteins and the possibility of different isozymic forms of the enzyme in the heart extract necessitated the use of a specific fractionation procedure to obtain a pure preparation. C1, a monoclonal antibody which reacts with the 60-kDa phosphodiesterase from bovine brain, was shown to cross-react with the heart enzyme. Thus, further purification of the heart enzyme was carried out by C1 antibody immunoaffinity chromatography.

The total calmodulin-binding protein sample was prepared from 2 kg of fresh bovine heart (Sharma, 1990). The total calmodulin-binding protein sample was applied to a C1 antibody–Sepharose 4B column (1.5 × 12.0 cm) which had been preequilibrated with buffer A containing 0.1 mM EGTA, 2 mM EDTA, and 10 mM 2-mercaptoethanol. The column was washed with 5–6 bed volumes of buffer A containing 0.1 mM EGTA, 2 mM EDTA, 10 mM 2-mercaptoethanol, and 0.5 M NaCl until no protein was detected in the column eluent by the dye-binding method (Bradford, 1976). Most of the protein of the original sample came through without binding or weakly bound, and had no phosphodiesterase activity. The calmodulin-dependent phosphodiesterase activity was eluted with buffer A containing 0.1 mM EGTA, 10 mM 2-mercaptoethanol, and 2.5 M $MgCl_2$. The fractions containing the highest activity of phosphodiesterase were pooled and dialyzed against buffer A containing 0.1 mM EGTA, 10 mM 2-mercaptoethanol, and 10% sucrose to remove $MgCl_2$. The dialyzed sample was concentrated to a least 1.0 mg of protein/mL by Amicon ultrafiltration.

Phosphorylation and Dephosphorylation of Calmodulin-Dependent Phosphodiesterase. The phosphorylation or dephosphorylation of phosphodiesterase was determined by the filter paper method described (Reimann et al., 1971) except 2% phosphoric acid was included in the 10% trichloroacetic acid solution used for filter paper washings. The reactions were carried out at 30 °C. The reaction mixtures for protein phosphorylation contained 100 mM Tris-HCl, pH 7.0, or 100 mM histidine buffer, pH 7.0, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1 mM [γ-^{32}P]ATP (100–200 cpm/pmol), and other components as described in the figure legends. Phosphorylated phosphodiesterase was dialyzed overnight with several changes against buffer A containing 0.1 mM EGTA, 10 mM 2-mercaptoethanol, and 10% sucrose to remove unreacted [γ-^{32}P]ATP in preparation for the dephosphorylation experiment. The dialyzed sample was used for dephosphorylation in a reaction mixture containing 100 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and other components as described in the figure legends.

Gel Electrophoresis and Autoradiography. Polyacrylamide gel electrophoresis in the presence of SDS was carried out (Laemmli, 1970). Coomassie Blue was used to visualize the protein bands on the gel. For autoradiography, the stained gel was dried on filter paper with a gel dryer and then exposed to X-Omat AR film in a Kodak X-Omatic cassette at −70 °C.

Other Methods. The calmodulin-dependent phosphodiesterase activity was measured as described (Sharma & Wang, 1979) except the assay mixture did not contain imidazole. The reaction mixture contained 40 mM Tris-HCl, 5 mM magnesium acetate, pH 7.5, 0.5 unit of 5′-nucleotidase, 1.2 mM cAMP, and other components as described in the figure legends in a total volume of 0.9 mL. Reaction was carried out at 30 °C for 30 min. One unit of phosphodiesterase is defined as the amount of enzyme which, when fully activated, hydrolyzes 1 μmol of cAMP/min at 30 °C. The activation of calmodulin-dependent phosphatase by preincubation with activating metal ions was carried out as described (Pallen & Wang, 1984).

Results

Purification and Purity. The purification procedure for calmodulin-dependent phosphodiesterase from bovine heart is described under Experimental Procedures. The last step, C1 antibody immunoaffinity column chromatography, resulted in greater than 100-fold purification. The recovery of the phosphodiesterase activity in this step was always greater than 90%. The specific activity of the purified samples ranged from 265 to 300 units/mg. The purification procedure is reproducible and rapid and yields a single polypeptide band of 59 kDa on SDS–polyacrylamide gel electrophoresis (Figure 1). Although the bovine heart and the 60-kDa subunit-containing bovine brain isozymes have similar immunological properties, the heart isozyme shows significantly higher affinity ($\simeq$6-fold) for calmodulin (Figure 2). The concentration of calmodulin required for half-maximal activation of bovine heart calmodulin-dependent phosphodiesterase and bovine brain 60-kDa phosphodiesterase isozyme was 0.15 and 0.90 nM, respectively (Figure 2).

Phosphorylation of Calmodulin-Dependent Phosphodiesterase. When purified calmodulin-dependent phosphodiesterase was incubated with cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP, the calmodulin-dependent phosphodiesterase was phosphorylated (Figure 3, lane 9). The phosphorylation of the phosphodiesterase was essentially inhibited by WIPTIDE, an inhibitor of cAMP-dependent protein kinase (results not shown). The phosphorylation of phosphodiesterase by cAMP-dependent protein kinase could be markedly increased by the use of histidine buffer, pH 7.0, instead of Tris-HCl buffer, pH 7.0 (Figure 3, lane 12). When the reaction was carried out in the presence of Ca^{2+} and calmodulin, the phosphorylation of phosphodiesterase was

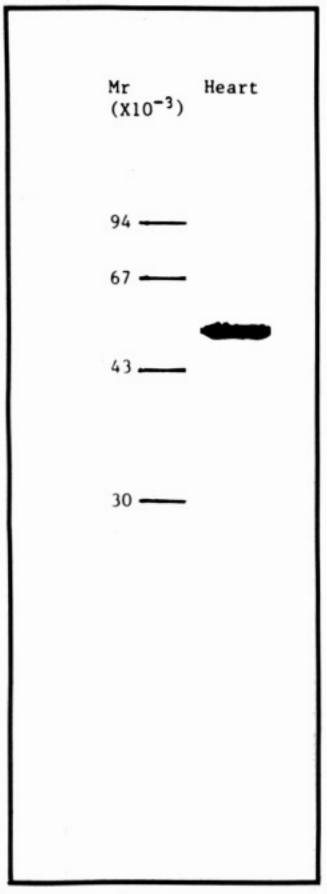

FIGURE 1: Analysis of purified calmodulin-dependent phosphodiesterase by SDS–polyacrylamide gel electrophoresis. Gel electrophoresis was carried out with 15 μg of phosphodiesterase as described under Experimental Procedures. Molecular weight standards: phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000).

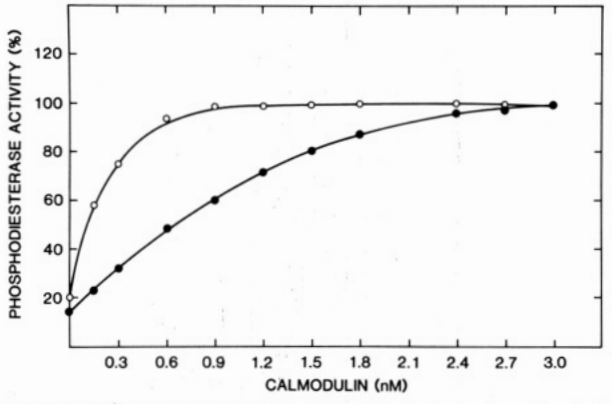

FIGURE 2: Differential stimulation of phosphodiesterase by calmodulin. Phosphodiesterase activity from bovine heart [(○) 48 ng/mL] and bovine brain 60-kDa isozyme [(●) 48 ng/mL] was measured in the presence of various concentrations of calmodulin in the presence of 0.1 mM Ca^{2+} as described under Experimental Procedures.

essentially eliminated (Figure 3, lane 14). The inhibition by Ca^{2+} and calmodulin appears to be substrate-directed (i.e., due to interaction of Ca^{2+}/calmodulin with the phosphodiesterase) since histone phosphorylation of cAMP-dependent protein kinase was not affected by Ca^{2+} and calmodulin (results not shown).

The rate of the phosphodiesterase phosphorylation in histidine buffer was compared with that in Tris-HCl buffer, pH 7.0 (Figure 4). The initial rate of phosphorylation was about 4-fold higher in histidine buffer than in Tris-HCl buffer with an increased level of phosphodiesterase phosphorylation.

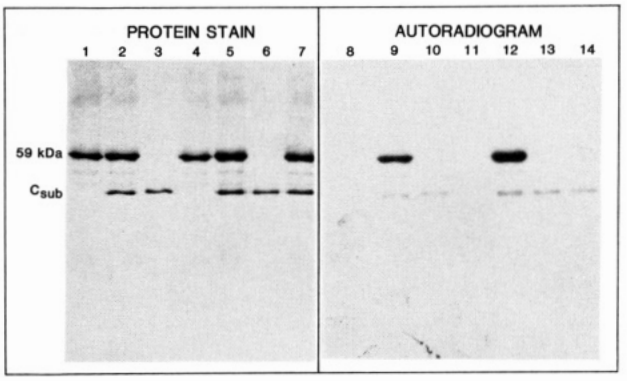

FIGURE 3: Phosphorylation of bovine heart calmodulin-dependent phosphodiesterase by cAMP-dependent protein kinase. Purified phosphodiesterase (380 μg/mL) was phosphorylated by cAMP-dependent protein kinase (25 μg/mL) for 40 min as described. Phosphorylation was carried out in Tris-HCl buffer, pH 7.0 (lanes 1–3), and histidine buffer, pH 7.0 (lanes 4–7). Reaction was also carried out in the presence of 0.1 mM Ca^{2+} and 150 μg/mL calmodulin (lane 7). After phosphorylation, an aliquot of each reaction was subjected to SDS gel electrophoresis. Lanes 1 and 4, purified calmodulin-dependent phosphodiesterase; lanes 2 and 5, purified calmodulin-dependent phosphodiesterase and cAMP-dependent protein kinase; lanes 3 and 6, cAMP-dependent protein kinase alone; lane 7, same as lane 5 with Ca^{2+} and calmodulin; lanes 8–14, respective autoradiographs of lanes 1–7.

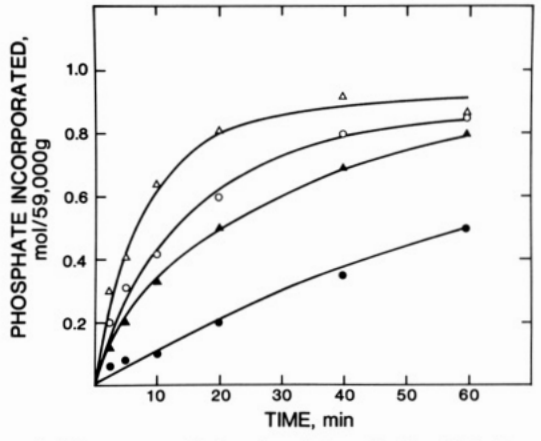

FIGURE 4: Time course of phosphorylation of calmodulin-dependent phosphodiesterase by cAMP-dependent protein kinase. Purified calmodulin-dependent phosphodiesterase (380 μg/mL) was phosphorylated by 25 μg/mL (●, ○) or 125 μg/μL (▲, △) cAMP-dependent protein kinase in 100 mM Tris-HCl buffer, pH 7.0 (●, ▲), or 100 mM histidine buffer, pH 7.0 (○, △), under standard assay conditions as described under Experimental Procedures.

Figure 4 shows that about 1 mol of phosphate was incorporated per mole of phosphodiesterase subunit after 60 min in histidine buffer whereas in Tris-HCl buffer reaction was slow. A higher concentration (5-fold) of cAMP-dependent protein kinase in the phosphorylation reaction resulted in a greatly enhanced rate of phosphorylation without any effect on the total phosphate incorporation: maximal phosphate incorporation was still about 1 mol/mol of subunit in both buffers (Figure 4).

The fact that other protein substrates, e.g., histone and phosphorylase kinase, were also phosphorylated by cAMP-dependent protein kinase at higher rates in histidine buffer than in Tris-HCl buffer suggests that the protein kinase is more active in histidine buffer (results not shown). A number of other buffers were therefore tested for their effect on phosphorylation of phosphodiesterase. Table I shows that histidine buffer appears to be the best buffer for the phosphorylation reaction. The rates of phosphodiesterase phosphorylation with other buffers were similar to that observed with Tris-HCl.

Table I: Effect of Various Buffers on the Phosphorylation of Calmodulin-Dependent Phosphodiesterase from Bovine Heart[a]

100 mM buffer, pH 7.0	phosphorylation of calmodulin-dependent phosphodiesterase
Tris-HCl	100
TES	120
MOPS	119
PIPES	115
histidine	185
imidazole	105
phosphate	109

[a] The bovine heart calmodulin-dependent phosphodiesterase (283 μg/mL) was incubated for 60 min with cAMP-dependent protein kinase (25 μg/mL) in various buffers as described under Experimental Procedures. The results are expressed as percent of phosphorylation of calmodulin-dependent phosphodiesterase.

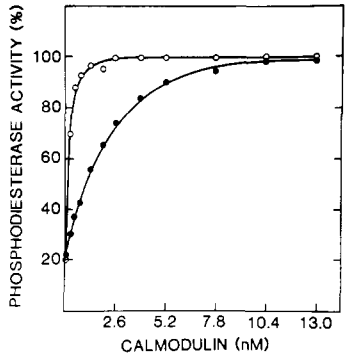

FIGURE 5: Effect of phosphorylation on the calmodulin concentration dependence of phosphodiesterase activation. The calmodulin-dependent phosphodiesterase (330 μg/mL) was phosphorylated by cAMP-dependent protein kinase (25 μg/mL) in histidine buffer, pH 7.0. After incubation for 60 min, an aliquot was removed and analyzed for phosphate incorporation. The amount of phosphate incorporated was 0.92 mol/mol of subunit of phosphodiesterase. A control reaction was carried out under identical conditions except buffer was substituted for [γ-^{32}P]ATP. Both the nonphosphorylated [(○) 40 ng/mL] and the phosphorylated [(●) 40 ng/mL] phosphodiesterases were analyzed for activity with various concentrations of calmodulin as indicated.

Effect of Phosphorylation on Phosphodiesterase Activity. The kinetic properties of the nonphosphorylated and phosphorylated phosphodiesterase were compared with cAMP and cGMP as substrates. Under all conditions, essentially similar activities were found for the phosphorylated and nonphosphorylated enzyme (results not shown). However, they showed markedly different affinities for calmodulin. Figure 5 shows that the amount of calmodulin required for 50% activation of the phosphorylated phosphodiesterase was 10-fold higher than that of the nonphosphorylated enzyme: 0.26 and 2.6 nM, respectively.

Phosphorylation also results in a difference in Ca^{2+} concentration of calmodulin required for activation of the phosphodiesterase at a constant concentration of calmodulin. At saturating levels of calmodulin (650 nM), the nonphosphorylated phosphodiesterase was activated 50% by 0.8 μM Ca^{2+} whereas the phosphorylated enzyme required 20.0 μM Ca^{2+} (Figure 6), suggesting that phosphorylation of the phosphodiesterase may modulate the Ca^{2+} sensitivity of the enzyme in vivo.

Dephosphorylation of Phosphodiesterase by Calmodulin-Dependent Phosphatase. Dephosphorylation of the phosphorylated phosphodiesterase was examined by using a calmodulin-dependent phosphatase (calcineurin) from bovine brain. The dephosphorylation reaction shown in Figure 7 was carried out with Ni^{2+}-stimulated phosphatase, and the reaction was calmodulin-dependent. Dephosphorylation correlated with

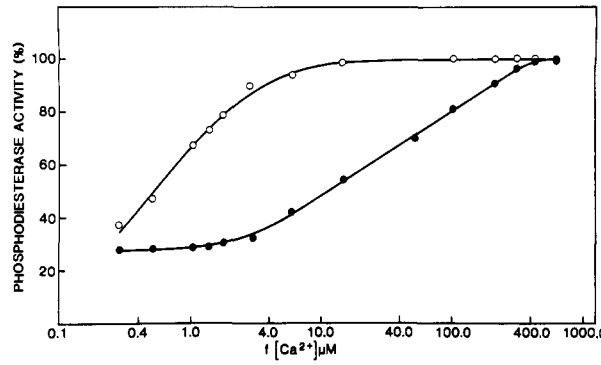

FIGURE 6: Effect of phosphorylation on the Ca^{2+} concentration dependence of calmodulin-dependent phosphodiesterase activation. Phosphorylation of calmodulin-dependent phosphodiesterase was carried out as described in Figure 5. Both the nonphosphorylated enzyme [(○) 40 ng/mL] and the phosphorylated enzyme [(●) 40 ng/mL] were assayed for phosphodiesterase activity as described under Experimental Procedures in the absence of imidazole and in the presence of 650 nM calmodulin, 5 mM magnesium acetate, and 1 mM EGTA. The free Ca^{2+} concentration in the EGTA-buffered solution was calculated as described previously (Huang et al., 1981).

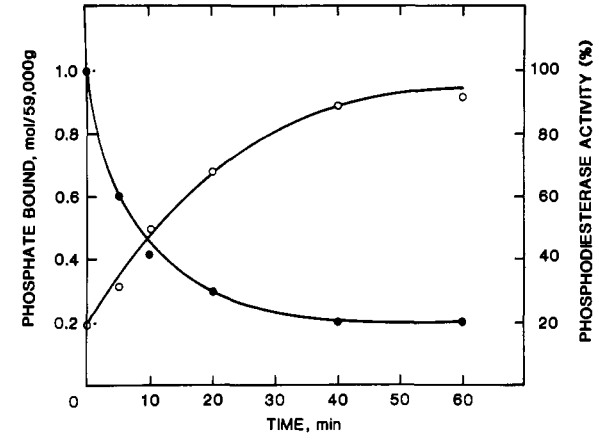

FIGURE 7: Time course of dephosphorylation by calmodulin-dependent phosphatase and calmodulin-dependent phosphodiesterase activity. Phosphorylation was carried out as described in Figure 5. Phosphorylated calmodulin-dependent phosphodiesterase was incubated at 30 °C in a reaction mixture containing 100 mM Tris-HCl, pH 7.0, 5 mM magnesium chloride, and 5 mM 2-mercaptoethanol with the addition of 0.1 mM Ni^{2+}, calmodulin-dependent phosphatase (20 μg/mL), and calmodulin (10 μg/mL). The calmodulin-dependent phosphatase was incubated with 1 mM Ni^{2+} at room temperature for 1 h prior to the phosphatase reaction. At the times indicated, aliquots were removed for assay of dephosphorylation (●) and phosphodiesterase activity (○) in the presence of 0.5 nM calmodulin with 0.1 mM Ca^{2+}.

an increase in the phosphodiesterase activity when assayed in the presence of 0.5 nM calmodulin, a concentration capable of stimulating the nonphosphorylated phosphodiesterase to about 80% of maximal activity. In contrast, basal and fully activated phosphodiesterase activities were not affected by dephosphorylation of the enzyme.

Discussion

Several calmodulin-dependent phosphodiesterase isozymes with different molecular weights and catalytic and regulatory properties have been purified to homogeneity and extensively characterized (Morill et al., 1979; Sharma et al., 1980, 1984; Kincaid & Vaughan, 1983; Kincaid et al., 1984; Shenolikar et al., 1985; LaPorte et al., 1979; Hansen & Beavo, 1982; Rossi et al., 1988). The present study describes a rapid purification procedure for the bovine heart enzyme utilizing C1 antibody immunoaffinity chromatography, enabling purification of the

bovine heart enzyme to apparent homogeneity. C1, a monoclonal antibody specific for the 60-kDa phosphodiesterase isozyme from bovine brain, was shown to cross-react with the heart enzyme. When the total calmodulin-binding fraction was applied to the C1 immunoaffinity column, all calmodulin-stimulated phosphodiesterase activity was bound, and this activity could be eluted by high concentrations of magnesium chloride. The result suggests that bovine heart may contain mainly one type of calmodulin-stimulated isozyme. This procedure is reproducible and suitable for large-scale preparation.

We have observed that heart calmodulin-dependent phosphodiesterase has a higher affinity ($\simeq$6-fold) for calmodulin than the bovine brain 60-kdA phosphodiesterase isozyme. Similar results were also reported (Mutus et al., 1985) when calmodulin dose dependence was examined by using the mixture of phosphodiesterase isozymes (63 and 60 kDa) from bovine brain and bovine heart. Hansen and Beavo (1986) also observed that heart phosphodiesterase has 10–20-fold higher affinity for calmodulin that the brain enzyme using cGMP as a substrate. This discrepancy in activation of phosphodiesterase may be due to different concentrations of phosphodiesterase as well as different substrates in the enzyme assay conditions of Hansen and Beavo (1986) in the two studies. The difference in calmodulin affinity exhibited by the heart and the brain enzyme may be related to the relative concentrations of calmodulin in the two tissues (Klee & Vanaman, 1982).

The bovine heart calmodulin-dependent phosphodiesterase can be phosphorylated by cAMP-dependent protein kinase. Interestingly, the phosphodiesterase phosphorylation rate could be increased severalfold by histidine buffer (Figures 3 and 4). The results suggest that an increase in phosphodiesterase phosphorylation rate is apparently due to the activation of the cAMP-dependent protein kinase by histidine. The inhibition of phosphorylation of calmodulin-dependent phosphodiesterase by Ca^{2+} and calmodulin suggests that the phosphorylation site may reside in or in proximity to the calmodulin-binding domain. The inhibition of phosphorylation of the brain 60-kDa phosphodiesterase isozyme by Ca^{2+} and calmodulin has also been demonstrated previously (Sharma & Wang, 1985, 1986b). These findings are in agreement with the finding of Malencik and Anderson (1983) that synthetic peptides which are substrates for cAMP-dependent protein kinase affect calmodulin binding.

There was no significant difference in the kinetic properties of phosphorylated and nonphosphorylated calmodulin-dependent phosphodiesterase from bovine heart; however, phosphorylation lowered the affinity for calmodulin and Ca^{2+} (Figures 5 and 6). The bovine brain calmodulin-dependent phosphodiesterase and smooth muscle myosin light chain kinase (Sharma & Wang, 1985, 1986b,c; Conti & Adelstein, 1981) have also been shown to undergo phosphorylation with a concomitant decrease in affinity for calmodulin. In all cases, the phosphorylated form of the enzyme retains its Ca^{2+} and calmodulin dependence, and at high concentrations of calmodulin, the enzyme activity is the same as that of the nonphosphorylated enzyme.

It has been shown that Ca^{2+} and calmodulin interact synergistically in the activation of calmodulin-dependent phosphodiesterase (Huang et al., 1981). At constant saturating concentration of calmodulin, phosphorylated phosphodiesterase was found to require a higher Ca^{2+} concentration for activation than the nonphosphorylated phosphodiesterase (Figure 6). Thus, this study suggests that the phosphorylation of the calmodulin-dependent phosphodiesterase may modulate the Ca^{2+} sensitivity of the enzyme. In addition, bovine brain 63- and 60-kDa calmodulin-dependent phosphodiesterase (Sharma & Wang, 1985, 1986b,c) and smooth muscle myosin light chain kinase (Conti & Adelstein, 1981) have also been shown to undergo phosphorylation, resulting in decreased affinities for Ca^{2+}. Dephosphorylation of calmodulin-dependent phosphodiesterase by calmodulin-dependent phosphatase correlated well with an increase in the enzyme's affinity for calmodulin. The dephosphorylation reaction is calmodulin-dependent whereas Ca^{2+}/calmodulin is capable of inhibiting the phosphorylation of phosphodiesterase (compare Figures 7 and 3). Results from the present study suggest that this regulatory mechanism can be fine-tuned by various interactions of Ca^{2+} and cAMP.

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Registry No. cAMP, 60-92-4; Ca, 7440-70-2; protein kinase, 9026-43-1; cyclic 3′,5′-nucleotide phosphodiesterase, 9040-59-9; histidine, 71-00-1.

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Cyclic Parathyroid Hormone Related Protein Antagonists: Lysine 13 to Aspartic Acid 17 [*i* to (*i* + 4)] Side Chain to Side Chain Lactamization

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ABSTRACT: Cyclization of parathyroid hormone related protein (7-34)amide [PTHrP(7-34)NH_2] via covalent bond formation between the ϵ-amino of Lys^{13} and the β-carboxyl of Asp^{17} yielded a 20-membered ring lactam. This analogue, [Lys^{13},Asp^{17}]PTHrP(7-34)NH_2, was 5-10-fold more potent than the linear parent peptide (K_b = 15 and 18 nM in PTH receptor binding assays, and K_i = 130 and 17 nM in PTH-stimulated adenylate cyclase assays in bovine renal cortical membrane and in human bone derived B10 cells, respectively). In contrast, a linear analogue in which charges in positions 13 and 17 were eliminated and other stereoisomers of the above-mentioned lactam in which either Lys^{13} and/or Asp^{17} were replaced by the corresponding D-amino acids were much less potent with regard to antagonist bioactivity than the parent peptide. The rationale for the design of the lactam as well as the conformational implications for the PTHrP sequence in light of reported models suggested for the 1-34 peptide are described. The potential use of conformationally constrained analogues for elucidating the "bioactive conformation" of antagonists and for the design of substantially simplified molecular structures for antagonists is discussed.

The purification, cloning, and structural elucidation of parathyroid hormone related protein (PTHrP) represents the successful conclusion of a long search for a tumor-secreted factor responsible for many cases of humoral hypercalcemia of malignancy (Moseley et al., 1987; Stewart et al., 1987; Mangin et al., 1988; Thiede et al., 1988). The new hormone, termed parathyroid hormone related protein (PTHrP), is structurally homologous to PTH in only a limited domain comprised of 8 out of 13 residues at the N-terminus (Suva et al., 1987) (see Figure 1). However, PTHrP interacts with what has been regarded conventionally as PTH receptors and stimulates actions similar to those caused by PTH with a potency comparable, for the most part, to that of PTH (Rodan et al., 1983; Stewart et al., 1983; Strewler et al., 1983).

Thus, PTH and PTHrP provide an unusual opportunity to conduct structure-activity studies based on two endogenous nonhomologous peptide hormones which interact with the same receptor to produce a very similar array of biological effects.

Like PTH, the synthetic N-terminal peptide, PTHrP(1-34)NH_2, is a full agonist in bone-derived (rat osteosarcoma cells, ROS 17/2.8 and UMR-106) and kidney-derived assays [bovine renal cortical membranes (BRCM) and opossum kidney (OK) epithelial cells (Horiuchi et al., 1987; Strewler et al., 1987; Juppner et al., 1988; Nissenson et al., 1988; Shigeno et al., 1988; Kemp et al., 1987; Rabbani et al., 1988; Pizurki et al., 1988)].

[Tyr^{34}]bPTH(7-34)NH_2 was the first PTH antagonist shown to be effective in vitro and in vivo (Mahaffey et al., 1979; Rosenblatt et al., 1988). Following the same rationale, truncation of 6 amino acid residues from the N-terminus of the PTHrP(1-34) agonist generated a potent antagonist, PTHrP(7-34)NH_2 (McKee et al., 1988). This peptide inhibited binding of radiolabeled PTH to receptors and anta-

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