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Isolation and Characterization of Bovine Cardiac Muscle cGMP-Inhibited Phosphodiesterase: A Receptor for New Cardiotonic Drugs

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SUMMARY

We have identified and highly purified a "low K_m" cAMP phosphodiesterase from bovine cardiac muscle. This phosphodiesterase was inhibited by low concentrations of cGMP and has, therefore, been temporarily designated as cGMP-inhibited phosphodiesterase. After a 16,000-fold increase in specific activity, the highly purified enzyme had a specific activity of 6 μmol/min-mg and contained three major polypeptides. Initial data indicated that all of these polypeptides were derived from a single common precursor by proteolysis. We used this enzyme preparation to generate polyclonal antisera and monoclonal antibodies directed against the "low K_m" phosphodiesterase. Immunoadsorption and immunoblot analysis allowed us to identify and isolate several molecular weight species of phosphodiesterase, including a larger form than previously reported for any purified low K_m phosphodiesterase. This large form of the enzyme had a subunit molecular weight of approximately 110,000 and was the only one seen in fresh extracts of cardiac muscle. Full catalytic activity was recovered in the phosphodiesterase-antibody complex and enzyme prepared by immunoprecipitation exhibited Michaelis-Menten kinetics for cAMP hydrolysis and for inhibition by cGMP. The K_m for cAMP hydrolysis was 0.15 μM and the K_i for cGMP inhibition of cAMP hydrolysis was 0.06 μM. This immunoprecipitation approach also allowed us to determine that the enzyme was phosphorylated on serine residues by cAMP-dependent protein kinase, and that the low K_m, cGMP-inhibited phosphodiesterase was selectively inhibited by several new cardiotonic agents. Milrinone, amrinone, and fenoximone were highly selective inhibitors of this isozyme, and the relative affinities of these inhibitors were consistent with their order of potency as positive inotropic agents. These studies suggest that the cGMP-inhibited phosphodiesterase is a receptor for several new cardiotonic drugs.

Most tissues and cells contain complex mixtures of phosphodiesterases that have very different physical and kinetic characteristics (1-3). This multiplicity of isozymes makes it difficult to determine the relative contributions of specific forms in crude tissue or cell preparations. In particular, there has been much controversy and uncertainty regarding the physical and catalytic characteristics, and the hormonal regulation of the so-called "low K_m," cAMP phosphodiesterases (4-9). We have identified and characterized a low K_m, cAMP phosphodiesterase in hypotonic extracts of bovine cardiac muscle. Although cGMP is poorly hydrolyzed by this enzyme, low concentrations of cGMP greatly inhibit hydrolysis of cAMP. For this reason, we have designated the enzyme as cGMP-inhibited phosphodiesterase. The presence of this cGMP-inhibited enzyme was masked by cGMP-stimulated phosphodiesterase in bovine heart extracts and in activity profiles after chromatography on DEAE cellulose (10). Kinetically similar low K_m, cGMP-inhibited activities have been reported previously in thymic lymphocytes (11), mutant S49 mouse lymphoma cells (12), human platelets (4, 13), bovine heart (10, 14), rat skeletal muscle (15) and rat liver (16). In addition, an insulin-sensitive cGMP-inhibited phosphodiesterase activity has been described in rat adipose tissue (9). Only the human platelet enzyme has been extensively purified and it is reported to be a 60-kDa protein (4).

Three new cardiotonic compounds, milrinone, amrinone, and fenoximone,[1] reportedly inhibit phosphodiesterase activity in crude cardiac enzyme preparations (17). Although no clear relationship has been established between phosphodiesterase inhibition and cardiotonic efficacy, fenoximone was shown to selectively inhibit one peak of low K_m phosphodiesterase activ-

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[1] Fenoximone was originally referred to in the literature as MDL 17043.

ABBREVIATIONS: PMSF, phenylmethylsulfonyl fluoride; PKI, heat-stable inhibitor of cAMP-dependent protein kinase; Staph A, heat-inactivated, formalin-fixed cells of the Cowan 1 strain of *Straphylococcus aureus*; RAM, rabbit anti-mouse IgG antiserum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IBMX, 3-isobutyl-1-methylxanthine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

ity after fractionation by DEAE cellulose (18). No specific receptor has been identified or purified, nor have selectivity studies been carried out with kinetically homogeneous preparations of the phosphodiesterases. The presence of multiple phosphodiesterase isozymes having kinetically different properties has complicated attempts to study the effects of inhibitors on cyclic nucleotide metabolism in cardiac tissues (3, 10, 17). Two of the major cardiac phosphodiesterases, the Ca^{2+}/calmodulin-activated phosphodiesterase and the cGMP-stimulated phosphodiesterase, are routinely purified to homogeneity in our laboratory by procedures which have been described previously (19, 20). In this paper we report the production and use of monoclonal antibodies for isolation and characterization of the third cardiac phosphodiesterase. Isolation of the three cardiac phosphodiesterases has allowed us to study the effects of various inhibitors on each of the enzymes.

These studies provide a physical and kinetic description of a newly isolated phosphodiesterase which is a receptor for several cardiotonic drugs. We also report on phosphorylation of the enzyme by cAMP-dependent protein kinase. Phosphorylation and inhibition by cGMP represent two potential mechanisms for regulating this low K_m cAMP phosphodiesterase.

Experimental Procedures

Materials

[2,8-^{3}H]cAMP (26 Ci/mmol) and [8-^{3}H]cGMP (6 Ci/mmol) were obtained from ICN Pharmaceuticals, and [γ-^{32}P]ATP (2900 Ci/mmol) were obtained from New England Nuclear. *Crotalus atrox* snake venom, PMSF, polyvinylpyrrolidone, cAMP, and cGMP were obtained from Sigma Chemical Co. DEAE-cellulose was obtained from Whatman, and DEAE-Sephadex A-25, cyanogen bromide-activated Sepharose 4B, and blue dextran 2000 were obtained from Pharmacia. Heat-inactivated and formalin-fixed cells of the Cowan 1 strain of *Staphylococcus aureas* were purchased from Calbiochem-Behring. Rabbit anti-mouse IgG antiserum was purchased from Miles Laboratories. High and low molecular weight standards for electrophoresis were obtained from Bio-Rad Laboratories. Female BALB/c mice were purchased from Fred Hutchinson Cancer Research Center. PKI and catalytic subunit of cAMP-dependent protein kinase were generously provided by the laboratory of Dr. E. G. Krebs at the University of Washington. Milrinone and amrinone were gifts from Sterling-Winthrop Research Institute, and fenoximone was a gift from Merrel Dow Research Institute. IBMX and papaverine were purchased from Sigma.

Methods

Preparation of bovine heart extracts. The same extraction procedure was used in both the large scale purification procedure and in the smaller scale purification on solid phase monoclonal antibodies. Bovine cardiac muscle was obtained from an abattoir and kept on ice. All subsequent operations were performed at 4°. Ventricular muscle was trimmed, sliced, passed through a coarse disk of a meat grinder, and homogenized in 2.5 volumes (w/v) of buffer containing 20 mM Tris-HCl, pH 7.8, and 15 mM 2-mercaptoethanol, in a Waring blender for 15 sec at high speed. Crystalline PMSF was added to a concentration of 0.2 mM during the homogenization. The homogenate was centrifuged at 4000 × g for 20 min and the resulting supernatant was filtered through glasswool and cheesecloth. The supernatant was then adjusted to pH 7.8 and PMSF was added to a final concentration of 0.4 mM.

Phosphodiesterase activity assay. Phosphodiesterase activity was determined as described previously (21), with minor modifications (22). Phosphodiesterase assays of immune pellets utilized resuspended Staph A-RAM-primary antibody pellets as enzyme samples, and Staph A-RAM pellets as enzyme blanks. Control pellet blanks represented less than 2% of the total tritium in an assay. Drug vehicles were tested at the highest concentrations used in the inhibitor studies and found to have no effect on the assays. The drugs studied did not inhibit the snake venom used in the assays. Rates of hydrolysis were linear over the range of times and dilutions used in the kinetic analysis.

Isolation of monoclonal antibody: cGMP-inhibited phosphodiesterase complexes for phosphorylation and inhibitor studies. Solid phase antibody reagents were prepared using monoclonal antibodies directed against the cGMP-inhibited phosphodiesterase, Staph A and RAM. Staph A control and antibody reagents were prepared and incubated with phosphodiesterase-containing samples as described in detail previously (22). The use of solid phase reagents allowed isolation of a specific phosphodiesterase by precipitation of phosphodiesterase-antibody complex. Since none of the antibodies inhibited enzyme activity, the phosphodiesterase was readily assayed on the resuspended immune pellet. Immune and control pellets were resuspended to the original sample volume in 20 mM Tris-HCl, pH 7.8 (4°) containing 50% glycerol and stored at −20° for use as a source of substrate in phosphorylation reactions and of enzyme in inhibitor studies.

Immune precipitation of other cardiac phosphodiesterases. Ca^{2+}/calmodulin-activated phosphodiesterase and cGMP-stimulated phosphodiesterase were immunoadsorbed from hypotonic extracts and from fractions collected after chromatography on DEAE cellulose as described above for cGMP-inhibited phosphodiesterase. Anti-Ca^{2+}/calmodulin-activated phosphodiesterase antibody and anti-cGMP-stimulated phosphodiesterase antibody (CGS-2) have been described previously (19, 23). Phosphodiesterase activities were measured on the antibody pellets.

Phosphorylation of cGMP-inhibited phosphodiesterase. cGMP-inhibited phosphodiesterase used in phosphorylation studies was isolated as described above. Phosphodiesterase-antibody-Staph A complex was incubated with heart kinase activity that was partially purified by chromatography on DEAE-cellulose. Phosphorylation reactions were initiated by the addition of Mg^{2+}[γ-^{32}P]ATP to 20 μl of phosphodiesterase (coupled to Staph A-antibody) and 15 μl of the heart kinase fraction, in a final volume of 100 μl. Reactions were carried out at 30° in 10 mM Hepes, pH 7.5, 0.5 mM dithiothreitol, 50 μM Na_3VO_4, 13 mM $MgCl_2$, and 0.16 mM ATP, and terminated by addition of 50 μl of ice-cold stop buffer (100 mM EDTA, 100 mM $NaPO_4$, and 10 μM $NaVO_4$). cAMP was added to a final concentration of 10 μM in some reactions to test for cAMP dependence of the heart kinase activity. PKI (24) was used to specifically inhibit the kinase activity. The specific activity of the [γ-^{32}P]ATP was 500 cpm/pmol in the phosphorylation reaction. The antibody-phosphodiesterase pellet was washed once as described above, and the phosphorylated protein was eluted by boiling in SDS-containing buffer. The sample was then loaded onto a 10% acrylamide gel and chromatographed by SDS-PAGE (25). ^{32}P was detected by autoradiography. Phosphorylations of similar preparations of antibody-complexed Ca^{2+}/calmodulin-activated phosphodiesterase and cGMP-stimulated phosphodiesterase were also tested by this method.

cGMP-inhibited phosphodiesterase purified by chromatography on blue dextran-Sepharose (described in Results) was concentrated 10- to 20-fold using a negative pressure protein dialysis/concentrator, and used in phosphorylation reactions. Enzyme sample containing approximately 1 μg of phosphodiesterase was incubated for 30 min at 30°, after initiation with Mg^{2+}/ATP, as described above. Kinase activity was provided by a trace contamination of protein kinase in the phosphodiesterase sample. PKI was used to specifically inhibit the contaminating kinase activity. These reactions were terminated by boiling in SDS sample buffer and analyzed by SDS-PAGE and autoradiography. Phosphoamino acid composition was determined by excision of the ^{32}P-containing bands, hydrolysis of the polypeptides by excess trypsin, and separation by high voltage paper electrophoresis as described previously (26, 27).

Purification of monoclonal antibodies. Monoclonal antibodies were purified using a Protein A Sepharose affinity chromatography procedure described previously (28), with minor modifications. Mouse

ascites fluid was centrifuged at 40,000 × *g* for 30 min at 4° and the supernatant fraction was adjusted to pH 8.2 (at 4°) with 1 M Tris buffer, pH 8.2 (at 4°). The sample was then applied dropwise to protein A·Sepharose resin, washed extensively with 10 mM Tris buffer, pH 8.2 (at 4°), and eluted with 10 mM citrate buffer, pH 4.5. The eluate was collected in tubes containing 1 M Tris buffer, pH 8.2 (at 4°). The resulting purified immunoglobulin protein was dialyzed against $NaHCO_2$, pH 8.0, 0.5 M NaCl coupling buffer for covalent attachment to cyanogen bromide-activated Sepharose.

Protein immunoblot analysis. cGMP-inhibited phosphodiesterase was purified by immunoadsorption from hypotonic extracts of bovine heart, using monoclonal antibodies directed against that isozyme. Immunoadsorption was performed using antibody coupled to cyanogen bromide-activated Sepharose 4B (affinity resin is described below), and control precipitations were performed using Sepharose coupled to Tris buffer instead of monoclonal antibody. Control and antibody-treated extracts were separated into supernatant and pellet fractions by centrifugation, and analyzed for phosphodiesterase activity and protein. The phosphodiesterase protein was eluted from the immune complex with 8 M urea, boiled in an SDS-containing buffer, and chromatographed by SDS-PAGE. The protein was then transferred to nitrocellulose and visualized by immunoblotting (29). Monoclonal antibodies and RAM secondary antibodies were diluted 1:1000 for use in this procedure. Following electrophoretic transfer of the proteins from the polyacrylamide gel to the nitrocellulose, polyvinylpyrrolidone was used to block nonspecific binding of the antibodies and ^{125}I-protein A to the nitrocellulose. Monoclonal antibodies were adsorbed to the cGMP-inhibited phosphodiesterase on the nitrocellulose and rabbit anti-mouse IgG sera and ^{125}I-protein A were subsequently complexed to the bound cGMP-inhibited phosphodiesterase antibodies. Autoradiography was carried out at −70° with an 8-hr exposure, in the presence of intensification screens.

Preparation of Sepharose affinity resins. Pharmacia blue dextran 2000 was coupled to cyanogen bromide-activated Sepharose 4B essentially as described by Pharmacia (manufacturer's instructions). Cyanogen bromide-activated Sepharose 4B (45 g) was swollen and washed extensively with 1 mM HCl and then washed quickly with $NaHCO_2$, pH 8.0, 0.5 M NaCl coupling buffer. Blue dextran 2000 (3.2 g) was dissolved in coupling buffer and incubated with the resin for 2 hr at room temperature. The resin was then washed extensively in the same buffer and blocked overnight with 1 M ethanolamine, pH 8.0, at 4°. The resin was subsequently washed with 3 volumes of 4 M guanidine hydrochloride, 2 M NaCl, 15 mM 2-mercaptoethanol, pH 7.5, and then equilibrated in buffer containing 40 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, and 15 mM 2-mercaptoethanol. Monoclonal antibodies purified from ascites fluid as described above were coupled to cyanogen bromide-activated Sepharose 4B using the same procedure with minor modifications. Five mg of IgG protein were coupled to 1 ml of washed resin and blocked overnight with 1 M Tris buffer at pH 8.0. The resin was then washed three times with alternating 0.1 M citrate buffer, pH 4.0, and 0.1 M Tris buffer, pH 8.0, both containing 0.5 M NaCl. cAMP and cGMP Sepharose resins were prepared by previously described methods (20).

Results

Large scale purification of the cGMP-inhibited phosphodiesterase from bovine cardiac muscle. Purified cGMP-inhibited phosphodiesterase was needed for biochemical characterization of the enzyme and for production of antibodies. Phosphodiesterase activity was extracted from 21 kg of bovine cardiac muscle as described in Experimental Procedures. The filtered supernatant was batch adsorbed onto 10 liters of DEAE cellulose. The resin was washed with approximately 70 liters of homogenization buffer containing 2 mM EDTA and 0.1 M NaCl, and eluted with a 20-liter linear NaCl gradient, 0.1 M–0.7 M, also in the presence of chelator. This procedure allowed removal of the Ca^{2+}/calmodulin phosphodiesterase during the wash step. Fractions were collected and then assayed for phosphodiesterase activity at 1 μM cAMP, and peak fractions containing both the cGMP-stimulated and cGMP-inhibited phosphodiesterases were pooled. Benzamidine was added to a final concentration of 15 mM and the sample was then passed sequentially through tandem cAMP and cGMP Sepharose resins. This step allowed the removal and purification of the cGMP-stimulated phosphodiesterase (20), and removal of other contaminating cyclic nucleotide-binding proteins. The phosphodiesterase activity which flowed through the resins was collected, magnesium chloride was added to a final concentration of 10 mM, and the pooled fractions were diluted to a conductivity of 7.0 mmho (at 4°) with 40 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, and 15 mM 2-mercaptoethanol. This sample was then mixed batchwise overnight with 120 ml of blue dextran-Sepharose resin and washed with 2 liters of the dilution buffer. The resin was poured into a 4.5 × 7.5-cm column and eluted with a 600-ml linear cAMP gradient (0–8 mM). Fig. 1 contains a typical blue dextran-Sepharose elution profile of phosphodiesterase activity assayed at 1.0 μM cAMP. This step provides an additional 100–200-fold purification of the cGMP-inhibited phosphodiesterase activity. The phosphodiesterase activity eluted from blue dextran-Sepharose was pooled and applied to a TSK-DEAE-5-PW anion exchange resin using a Waters HPLC system. This work was done at room temperature, but the fractions were collected at 4° in an adjacent cold box. The applied sample was centrifuged for 10 min in a Beckman Airfuge at full speed prior to the HPLC step. The column was loaded and washed in 5 mM Tris-HCl, pH 7.5, 0.25 M NaCl, and eluted with 0.33 M NaCl. A second peak containing a small amount of phosphodiesterase activity was eluted with 0.5 M NaCl. Fig. 2 demonstrates a typical elution profile. The results obtained using this procedure are shown in Table 1. The enzyme was purified 16,000-fold with a yield of approximately 5% and a specific activity of 6 μmol of cAMP hydrolyzed/min-mg.

The procedure produced a highly purified phosphodiesterase;

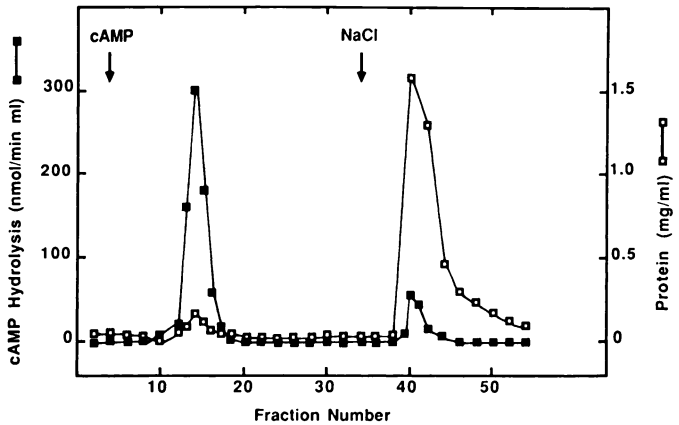

Fig. 1. Blue dextran-Sepharose profile of cGMP-inhibited phosphodiesterase activity. Phosphodiesterase activity that flowed through the cyclic nucleotide affinity resins was applied batchwise to blue dextran-Sepharose overnight at 4° and eluted with a cAMP gradient as described in Results. Phosphodiesterase activity was assayed at 1 μM cAMP (■), and protein was assayed using the Bradford assay (30) with bovine serum albumin as the protein standard (□). A second peak containing most of the bound protein and a small amount of phosphodiesterase activity was eluted with 0.75 M NaCl and 10 mM cAMP.

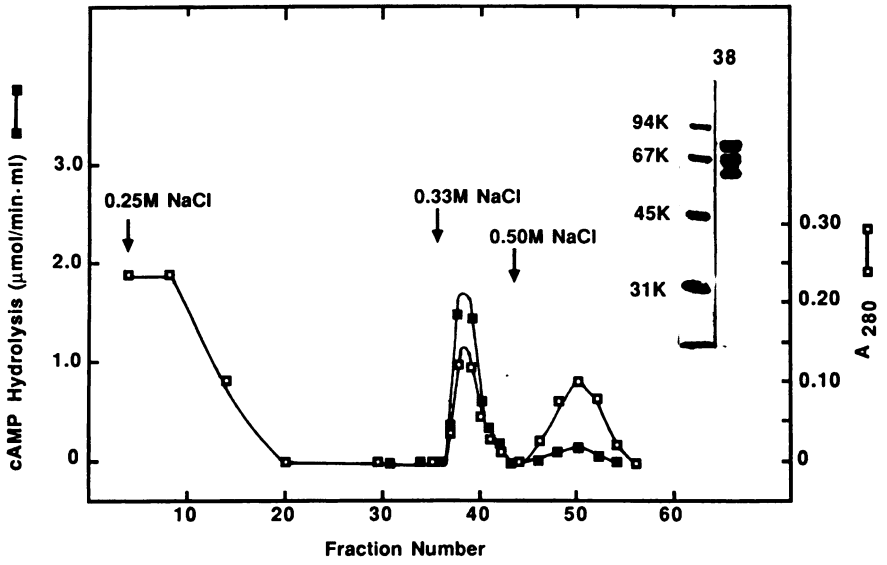

Fig. 2. HPLC profile of cGMP-inhibited phosphodiesterase activity. Phosphodiesterase activity eluted from blue dextran-Sepharose was applied to a TSK-DEAE anion exchange column and eluted stepwise with sodium chloride, as described in Results. Phosphodiesterase activity was assayed at 1 μM cAMP (■), and protein was assayed using absorbance at 280 nm (□). The cGMP-inhibited phosphodiesterase is both purified and concentrated by this procedure. The *gel lanes* included in the figure contain Coomassie-stained protein standards and proteins from fraction 38, after SDS-PAGE. Protein pooled from the first peak of phosphodiesterase activity was used to produce antibodies directed against the cGMP-inhibited phosphodiesterase.

TABLE 1

Purification of a "low K_m," cAMP phosphodiesterase from bovine cardiac muscle (21 kg of cardiac muscle)

Sample	Total protein	Specific activity	Fold purification	% Recovery
	mg	*μmol/min-mg*		
Ab-treated extract[a]	517,500	$0.38\ 10^{-3}$	1	100
DEAE/cNMP-Sepharose[b]	12,575	$8.2\ 10^{-3}$	22	52
Blue dextran Sepharose	10	1.5	3,947	8
HPLC-DEAE	1.6	6.0	15,789	5

[a] cGMP-stimulated and Ca^{2+}/calmodulin-activated phosphodiesterases removed by antibody adsorption.
[b] DEAE-cellulose pooled fractions after passage through cAMP and cGMP Sepharose resins.

however, most preparations contained predominantly an 80-kDa polypeptide which slowly degraded to 67- and 60-kDa polypeptides (Fig. 2, *inset*). Increasing amounts of the lower molecular weight polypeptides were observed with time in both blue dextran-Sepharose and HPLC-DEAE pooled fractions (data not shown). Some improvement in the stability of the blue dextran-Sepharose-purified protein was obtained by adjusting the buffer to include: 10 μM leupeptin, 10 μM pepstatin A, 2 mM *ortho*-phenanthroline, 2 mM EGTA, and 3 mM EDTA. HPLC-purified, cGMP-inhibited phosphodiesterase contained several enzyme fragments which were eluted from the TSK-DEAE resin by 0.33 M NaCl. More recently we have found that the 110-kDa enzyme described below is eluted by a higher salt concentration. This difference in binding to DEAE by partially degraded enzyme may explain the broad peaks of "low K_m," cAMP phosphodiesterase activity commonly observed after chromatography on DEAE cellulose. Use of the protease inhibitors allowed us to obtain a greater proportion of the enzyme in the higher molecular weight forms; however, we have not yet obtained a stable, homogeneous preparation of the enzyme by this procedure. Methods were needed for identifying cGMP-inhibited phosphodiesterase protein and isolating a stable enzyme. For these reasons, the HPLC-purified phosphodiesterase shown in Fig. 2 was used as antigen for the production of polyclonal antisera and monoclonal antibodies.

Production of antibodies. Polyclonal antisera were generated by subcutaneous injections of five BALB/c mice with 50 μg of HPLC-purified cGMP-inhibited phosphodiesterase protein (purification of antigen is described in detail in Results; see Fig. 2 *inset*) in Freund's complete adjuvant. The mice were boosted intraperitoneally with antigen in Freund's incomplete adjuvant 8 weeks after the primary immunization. Antibody titer was measured 21 days after boosting the mice. All five mice developed higher titer antisera against cGMP-inhibited phosphodiesterase activity. The polyclonal antisera from these mice precipitated 100% of the enzyme activity in solid phase immune precipitation assays. One of the mice was boosted with 50 μg of antigen on days 4, 3, and 2 prior to the fusion, based on a report that such a boosting schedule greatly increased the percentage of antibody-producing hybridomas, when immunizing with soluble antigens (31). Spleen cells from the mouse were fused with Sp2/0 cells derived from the cloned BALB/c mouse myeloma line P3-X63-Ag8, to generate monoclonal antibody-producing hybridomas by methods modified from the orginal procedure of Kohler and Milstein (32), as described previously (33). Hybridoma colonies were screened for production of anti-cGMP-inhibited phosphodiesterase antibodies by immunoadsorption, and phosphodiesterase activity was measured on the immune pellets as described in Experimental Procedures. Five colonies of antibody-producing hybridomas were orginally identified from 150 growing colonies (in 960 wells). During subsequent culturing and subculturing, two of the original five were lost. All three of the remaining cell lines (CGI-2, 4, and 5) produced IgG1 antibodies that were useful for immune precipitations and protein immunoblotting, and none of the antibodies inhibited phosphodiesterase activity.

Preliminary characterization of bovine heart cGMP-inhibited phosphodiesterase. Monoclonal antibodies directed against the cGMP-inhibited phosphodiesterase were used to rapidly isolate the enzyme from heart extracts and from partially purified samples. Binding of the enzyme to solid phase antibodies did not inhibit enzyme activity or alter enzyme inhibition by several highly selective inhibitors (22). Enzyme isolated by this method had a K_m of 0.15 μM for cAMP hydrol-

ysis and a K_i of 0.06 μM for cGMP inhibition of cAMP hydrolysis. The K_i for cGMP inhibition was determined by the method of Dixon (34). Reciprocal plots of the kinetic data were linear, and cGMP was a competitive inhibitor of cAMP hydrolysis. The rate of cGMP hydrolysis at 1 μM was 8% of the rate measured for cAMP hydrolysis at 1 μM.

Protein immunoblot analysis of the antibody-purified cGMP-inhibited phosphodiesterase demonstrated an apparent subunit molecular weight of 110,000 (Fig. 3, lane B). Previously reported subunit molecular weights for purified low K_m phosphodiesterases range from 50,000 to 60,000 (4–6). In our studies, cardiac cGMP-inhibited phosphodiesterase prepared by standard fractionation techniques was rapidly degraded by endogenous heart proteases. Extract incubated overnight at room temperature contained partially degraded enzyme, with an apparent subunit molecular weight of 80,000 (Fig. 3, lane C). No detectable protein was adsorbed from the fresh extract by the control Sepharose beads (Fig. 3, lane D). Preliminary observations in our laboratory suggested that the partially degraded enzyme preparations were catalytically active but contained a heterogeneous population of catalytic sites with different affinities for substrates and inhibitors, which greatly complicates the interpretation of inhibitor and kinetic studies.

Phosphorylation of cGMP-inhibited phosphodiesterase. cGMP-inhibited phosphodiesterase purified by chromatography on blue dextran-Sepharose, concentrated by ultrafiltration, and stored in the protease inhibitor cocktail (described above) was isolated in a high molecular weight form and was more slowly degraded by contaminating proteases. This preparation of enzyme contained predominantly 110-kDa phosphodiesterase with traces of 80- and 60-kDa fragments,[2] and was used in phosphorylation experiments. Phosphorylation was detected in the absence of exogenous kinase (Fig. 4A). This phosphorylation was inhibited by the heat-stabile inhibitor of cAMP-dependent protein kinase, and was due to a trace-contaminating kinase, since no kinase activity was detected in subsequent experiments using antibody-purified cGMP-inhibited phosphodiesterase. Phosphorylation was observed on serine residues of the 110-kDa polypeptide (Fig. 4C).

Monoclonal antibody-purified enzyme was used as a substrate in phosphorylation reactions with partially purified heart protein kinase activity as described in Experimental Procedures, or with purified catalytic subunit of cAMP-dependent protein kinase. This approach was used to confirm that the phosphorylated protein was cGMP-inhibited phosphodiesterase. Kinase activity was extracted from 100 g of heart muscle and chromatographed on DEAE cellulose using a procedure identical to that described for the phosphodiesterases. cGMP-inhibited phosphodiesterase was phosphorylated by cAMP-dependent protein kinase, and the phosphorylation was inhibited by the heat-stabile inhibitor of cAMP-dependent protein kinase (Fig. 4B). The protein phosphorylated on the monoclonal antibody had a subunit molecular weight of 110,000, identical to the size obtained by protein immunoblot analysis. Preliminary experiments have failed to detect significant changes in cAMP or cGMP hydrolysis as a result of phosphorylation of the enzyme. cGMP-stimulated phosphodiesterase and Ca^{2+}/calmodulin-activated phosphodiesterase were also isolated from bovine cardiac muscle in complexes

[2] S. A. Harrison, B. Gallis, and J. A. Beavo, unpublished observations.

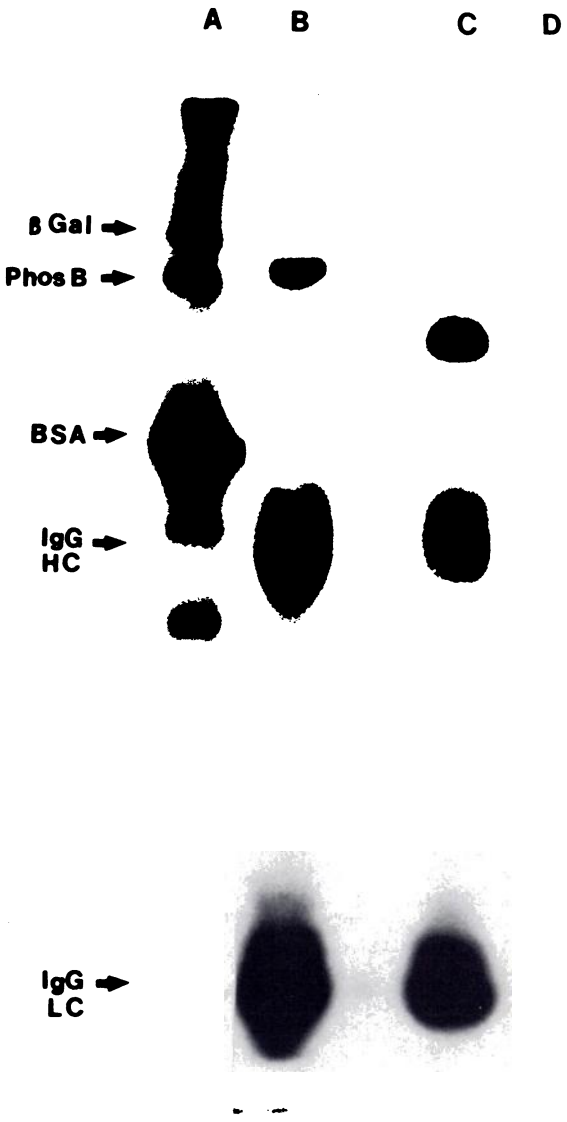

Fig. 3. Protein immunoblot analysis of purified cGMP-inhibited phosphodiesterase subunit molecular weight. cGMP-inhibited phosphodiesterase was purified by immunoadsorption from hypotonic extracts of bovine heart, using monoclonal antibodies directed against that isozyme (Experimental Procedures). Immunoadsorption was performed using antibody coupled to cyanogen bromide-activated Sepharose 4B, and control precipitations were performed using Tris buffer coupled to Sepharose without monoclonal antibody. Control and antibody-treated extracts were separated into supernatant and pellet fractions by centrifugation and analyzed for phosphodiesterase activity and protein. Phosphodiesterase protein was eluted from the immune complex, chromatographed by SDS-PAGE, and then transferred to nitrocellulose for immunoblotting. Iodinated protein standards were chromatographed by SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography (*lane A*). A specific band of cGMP-inhibited phosphodiesterase protein with an apparent molecular weight of 110,000 was adsorbed from freshly prepared hypotonic extract and detected on the antibody pellet (*lane B*). Some IgG heavy and light chains were also eluted from the Sepharose beads and detected by the RAM secondary antibody. In hypotonic extracts incubated overnight at room temperature prior to adsorption onto antibodies, the cGMP-inhibited phosphodiesterase was degraded to an 80-kDa fragment (*lane C*). Tris-Sepharose, used as a control for nonspecific adsorption to the Sepharose beads, did not precipitate any antibody-reactive protein (*lane D*).

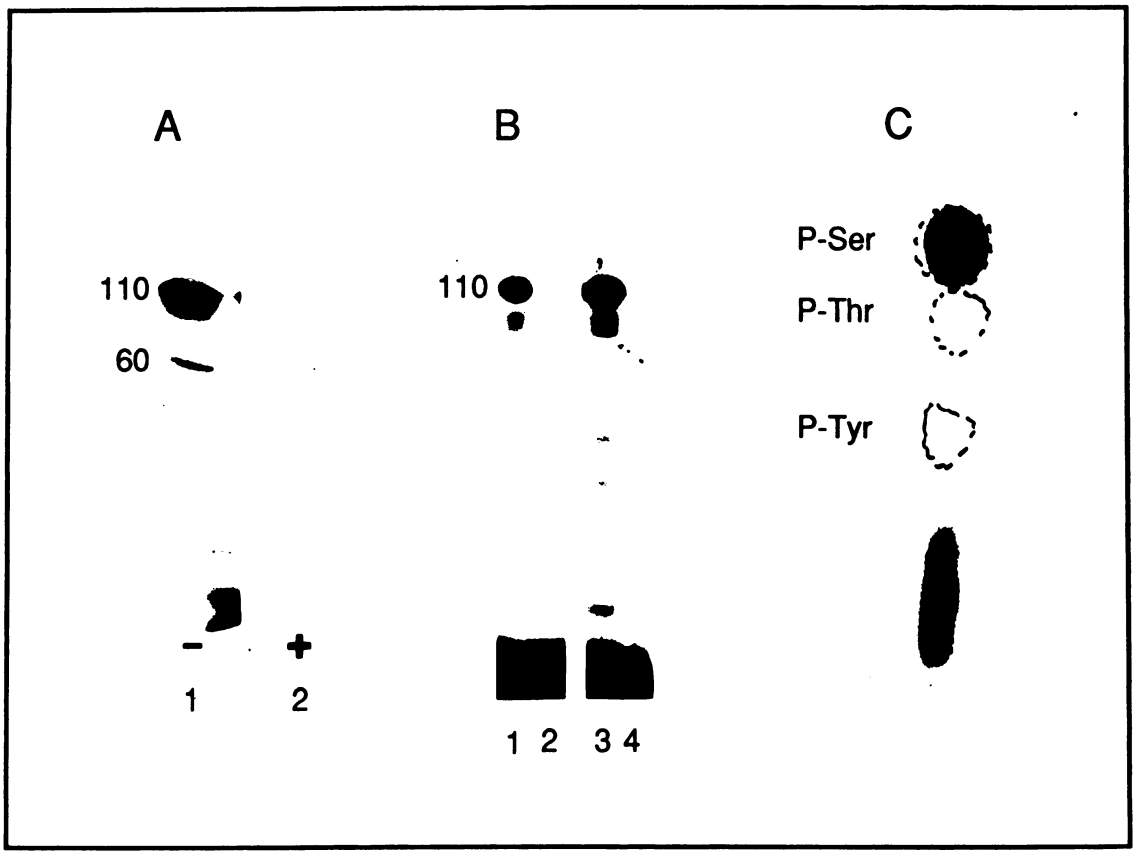

Fig. 4. Phosphorylation and phosphoamino acid analysis of cGMP-inhibited phosphodiesterase. Phosphorylation reactions described in Experimental Procedures were terminated by boiling in Laemmli sample buffer and the proteins were chromatographed by SDS-PAGE. A. The 110-kDa polypeptide obtained from a highly purified preparation of cGMP-inhibited phosphodiesterase was phosphorylated by a contaminating kinase activity (*lane 1*), and that phosphorylation was inhibited by PKI (*lane 2*). B. Phosphodiesterase-monoclonal antibody complex was incubated with heart kinase activity that had been fractionated on DEAE-cellulose (*lanes 1 and 2*), or with purified catalytic subunit of cAMP-dependent protein kinase (*lanes 3 and 4*). Heat-stabile inhibitor of cAMP-dependent protein kinase was found to specifically inhibit the kinase activity (*lanes 2 and 4*). C. After localization of the 110-kDa polypeptide by Coomassie staining and autoradiography, the protein band was excised and the phosphoamino acid content was determined as described in Experimental Procedures. An autoradiogram after high voltage paper electrophoresis of the ^{32}P-containing amino acids is shown.

with appropriate monoclonal antibodies as described in Experimental Procedures. No phosphorylation of antibody-purified cGMP-stimulated phosphodiesterase or Ca^{2+}/calmodulin-activated phosphodiesterase was observed under these conditions.

Phosphodiesterase inhibitor studies. Milrinone, amrinone, fenoximone, RO 20-1724, IBMX, and papaverine inhibitions of homogeneous cGMP-inhibited phosphodiesterase, Ca^{2+}/calmodulin-activated phosphodiesterase, and cGMP-stimulated-phosphodiesterase were measured at low concentrations of cAMP. Bovine heart cGMP-stimulated phosphodiesterase and Ca^{2+}/calmodulin-activated phosphodiesterase were assayed in the presence of cGMP and calcium/calmodulin, respectively. Under these conditions, both enzymes contribute significantly to the hydrolysis of low concentrations of cAMP (10). IC_{50} curves for inhibition of the three heart phosphodiesterases illustrate a selective and a relatively nonselective inhibitor (Fig. 5). Only the cGMP-inhibited phosphodiesterase was inhibited by low concentrations of the new cardiotonic drugs, in this case fenoximone (Fig. 5A). Papaverine exhibited relatively little selectivity for any heart phosphodiesterase (Fig. 5B). All other soluble cardiac phosphodiesterase activity was measured after removal of cGMP-inhibited phosphodiesterase, cGMP-stimulated phosphodiesterase, and Ca^{2+}/calmodulin-activated phosphodiesterase from the extracts by immunoadsorption using monoclonal antibodies specific for each form. This phosphodiesterase activity remaining in the extract after antibody treatment was not selectively inhibited by any of the drugs tested.

Inhibitor data were analyzed by the method of Dixon (34) in order to determine the affinity of each drug for the phosphodiesterases and the nature of the inhibition. Linear reciprocal plots of the data indicated that milrinone inhibited a single class of catalytic sites in preparations of cGMP-inhibited phosphodiesterase (Fig. 6). The plots also indicated competitive inhibition of cAMP hydrolysis by milrinone, presumably at the catalytic site. Linear, competitive Dixon plots were observed for all of the drugs tested. When cGMP-inhibited phosphodiesterase was isolated from a less rapidly prepared enzyme source, nonlinear Dixon plots were observed, suggesting catalytic site heterogeneity, i.e., partial degradation of the monoclonal antibody-bound, cGMP-inhibited phosphodiesterase (data not shown). Table 2 presents a summary of the data obtained from the Dixon plots. Milrinone and fenoximone exhibited at least 100-fold greater affinity for the cGMP-inhibited phosphodiesterase than for the other heart phosphodiesterases. IBMX and papaverine were potent but relatively nonselective inhibi-

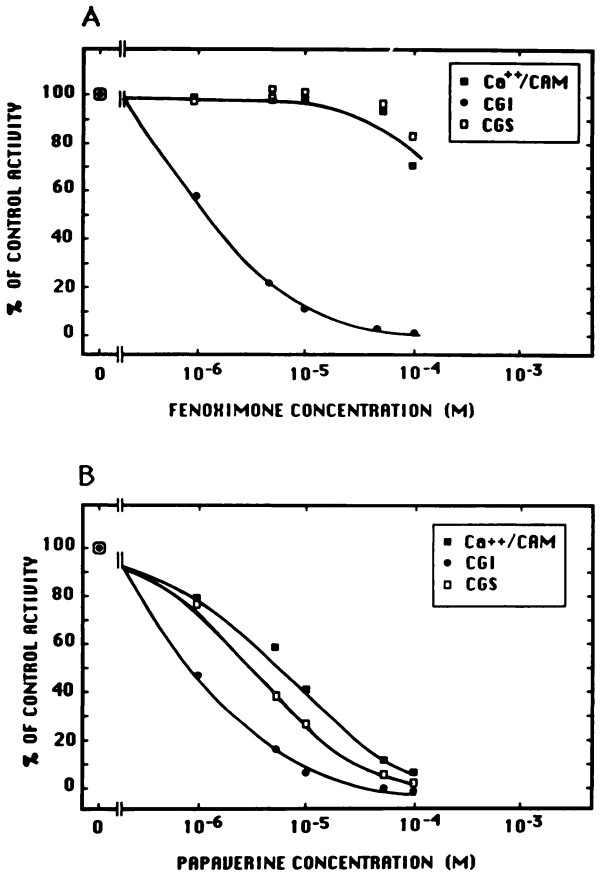

Fig. 5. Drug inhibition curves comparing fenoximone and papaverine inhibition of cardiac phosphodiesterases. cGMP-inhibited phosphodiesterase immunoadsorbed from bovine cardiac muscle hypotonic extracts was prepared as described in Experimental Procedures and used as an enzyme source in all of the inhibitor studies. Immunoadsorbed cGMP-inhibited phosphodiesterase (●), and purified Ca^{2+}/calmodulin-activated phosphodiesterase (■) and cGMP-stimulated phosphodiesterase (□) were assayed for activity at 0.35 μM cAMP over a wide range of (A) fenoximone (data in panel A replotted from Ref. 22 for purposes of comparison) or (B) papaverine concentrations. cGMP-stimulated phosphodiesterase was assayed in the presence of 1 μM cGMP. Ca^{2+}/calmodulin-activated phosphodiesterase was assayed in the presence of saturating calcium and calmodulin. Data were plotted as percentages of control activities measured in the absence of inhibitor. Fenoximone selectively inhibited the heart cGMP-inhibited phosphodiesterase; however, papaverine was a relatively potent inhibitor of all the phosphodiesterases.

tors of all heart phosphodiesterases. RO 20-1724, a potent inhibitor of some crude preparations of low K_m, cAMP phosphodiesterase activity (36, 37), was not a good inhibitor of the cGMP-inhibited phosphodiesterase from bovine cardiac muscle. These results demonstrate that milrinone, amrinone, and fenoximone are highly selective inhibitors of bovine cardiac muscle cGMP-inhibited phosphodiesterase, and suggest this enzyme as a site of action for these cardiotonic drugs.

Discussion

We have identified and purified a "low K_m," cAMP phosphodiesterase from bovine cardiac muscle. The procedure described in this work resulted in approximately 16,000-fold purification of the phosphodiesterase activity. The enzyme had a specific activity of 6 μM/min-mg for cAMP hydrolysis. Although this procedure did not result in a stable, homogeneous preparation

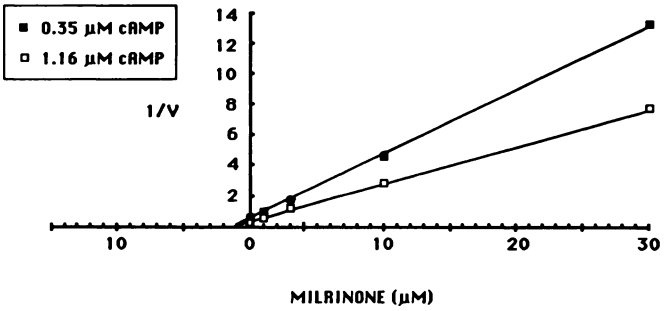

Fig. 6. Dixon plot of data from milrinone inhibition of cardiac cGMP-inhibited phosphodiesterase. cGMP-inhibited phosphodiesterase was immunoadsorbed from hypotonic extracts of bovine heart and assayed for activity at 0.35 μM (■) and 1.16 μM (□) cAMP, over a wide range of milrinone concentrations, as described in the legend to Fig. 5. These data were analyzed using reciprocal plots according to the method of Dixon (34). Lines were fitted to the data by least squares linear regression analysis. Data points in this representative plot are the average of duplicate points. A wide range of drug concentrations was used to demonstrate the linearity of the plot. Similar kinetics were observed in experiments with more points over a narrow range of drug concentrations. The linearity of the plots indicated that the rapidly prepared cGMP-inhibited phosphodiesterase was homogeneous for drug binding.

of the enzyme, the highly purified protein was used to produce polyclonal and monoclonal antibodies directed against the cGMP-inhibited phosphodiesterase. The mouse polyclonal antisera directed against the cGMP-inhibited phosphodiesterase did not crossreact with purified cGMP-stimulated phosphodiesterase or Ca^{2+}/calmodulin-activated phosphodiesterase from bovine heart, or partially purified low K_m cAMP phosphodiesterase activity from either bovine lung or canine kidney (22). The immunological data, and preliminary kinetic and physical data, argue that the cGMP-inhibited phosphodiesterase is a distinct isozyme.

Antibodies directed against the cGMP-inhibited phosphodiesterase did not inhibit catalytic activity, and they were useful for protein immunoblot analysis. The monoclonal antibodies were used to isolate and identify a larger form of low K_m, cAMP phosphodiesterase than has been detected previously. Antibody-purified enzyme had an apparent subunit molecular weight of 110,000, a K_m of 0.15 μM for cAMP hydrolysis, and a K_i of 0.06 μM for cGMP inhibition of cAMP hydrolysis. Three different monoclonal antibodies precipitated both protein and activity from purified enzyme samples, strongly implying that the catalytic site was part of the same 110-kDa protein and not a trace contaminant.

cGMP-inhibited phosphodiesterase was phosphorylated *in vitro* on serine residues by bovine heart cAMP-dependent protein kinase. Although no significant alteration of catalytic activity was observed under conditions of phosphodiesterase phosphorylation, we have not looked for more subtle modifications, such as those involving more than one protein kinase, or those involving phosphodiesterase interactions with other proteins. Phosphorylation and inhibition by cGMP are possible mechanisms for regulating this cardiac cAMP phosphodiesterase.

Inhibitor data support the hypothesis that several new cardiotonic agents share a common mechanism of action: highly selective inhibition of a single phosphodiesterase isozyme present in cardiac tissues. We have identified, isolated, and characterized a receptor for those drugs, and have measured its affinity for a variety of antagonists. The relative affinities of

TABLE 2

Inhibitor constants for cardiac phosphodiesterases[a]

Reciprocal plots were analyzed for all of the drugs tested, and inhibitory constants were determined for each heart phosphodiesterase. The X intercept (X_i) for each Dixon plot was determined using least squares linear regression analysis of the data. The inhibitor constants were then calculated from the equation: $K_i = -X_i/(1 + [s]/K_m)$ (34). The substrate concentrations used were 0.35 μM and 1.16 μM cAMP. K_m values used for the cardiac enzymes were 0.15, 30, and 160 μM, respectively, for the cGMP-inhibited phosphodiesterase, cGMP-stimulated phosphodiesterase, and Ca^{2+}/calmodulin-activated phosphodiesterase (20, 35). K_i values obtained for the latter two enzymes by this method were not greatly affected by minor variations in substrate concentration or K_m values. cGMP-stimulated phosphodiesterase and Ca^{2+}/calmodulin-activated phosphodiesterase were assayed in the presence of the activators as described in Fig. 5. Under these conditions each isozyme contributes significantly to cAMP hydrolysis at the low concentrations assayed. Data are expressed ± standard deviation (n = 6–9 for cGMP-inhibited phosphodiesterase and n = 2–4 for cGMP-stimulated phosphodiesterase and Ca^{2+}/calmodulin-activated phosphodiesterase).

	Milrinone	Amrinone	Fenoximone	IBMX	Papaverine	RO 20-1724
CGI-PDE	0.26 ± 0.10	7.9 ± 2.5	1.5 ± 0.6	1.3 ± 0.5	0.66 ± 0.31	62 ± 15
Ca^{2+}/CAM-PDE	48 ± 1	880 ± 40	240 ± 10	6.7 ± 2.5	12 ± 4	240 ± 71
CGS-PDE	180 ± 10	400 ± 80	430 ± 160	14 ± 3	4.5 ± 2.5	300 ± 45

[a] Inhibitor concentration × 10^6 M.

these agents, milrinone > fenoximone > amrinone, were consistent with values reported for their positive inotropic effects. Milrinone, fenoximone, and amrinone increase contractility with little effect on heart rate in several species (17, 38). Compounds such as IBMX and papaverine, which are potent inhibitors of cGMP-inhibited phosphodiesterase and other heart phosphodiesterases, exhibit both positive inotropic and chronotropic effects (39). These data suggest that the pool of cAMP regulated by the cGMP-inhibited phosphodiesterase has a major role in the regulation of cardiac muscle contractility. They also imply either that heart phosphodiesterases regulate different pools of cAMP or that some of the phosphodiesterase inhibitors have several sites of action. Additional evidence is needed to demonstrate that the cGMP-inhibited phosphodiesterase is the only site of action for these cardiotonic drugs. The new cardiotonic drugs may be useful not only as therapeutic agents in the treatment of heart disease, but also as probes for determining the role of cGMP-inhibited phosphodiesterase in regulating physiological responses.

The procedure for rapid isolation of cGMP-inhibited phosphodiesterase described in this work should be useful for screening new drugs and for characterization of the phosphodiesterase molecule. The present studies demonstrate the utility of this approach for detection of phosphorylation *in vitro*, for identification of highly selective inhibitors, and for detection of a high molecular weight form of the enzyme. The antibodies and inhibitors should also be useful for studying the relationship between the cardiac cGMP-inhibited phosphodiesterase and the insulin-sensitive cAMP phosphodiesterase in rat adipocytes, and the low K_m phosphodiesterase in the mutant S49 cell line, K30a. Both of the latter enzymes are immunologically and pharmacologically similar to the bovine heart enzyme (40). The immunoadsorption method can also be utilized to study Ca^{2+}/calmodulin-activated phosphodiesterase and cGMP-stimulated phosphodiesterase in cardiac and other tissues.

Three major phosphodiesterase isozymes are present in bovine cardiac muscle: Ca^{2+}/calmodulin-activated phosphodiesterase, cGMP-stimulated phosphodiesterase, and cGMP-inhibited phosphodiesterase. All three enzymes can make significant contributions to cAMP hydrolysis at physiological nucleotide concentrations, in the presence or absence of the appropriate regulatory molecules. cGMP-stimulated and cGMP-inhibited phosphodiesterases exhibit similar sensitivity to cGMP (20). The presence of both enzymes in heart and their potential for regulation by cGMP suggest a possible role for cGMP as a regulatory switch, activating one enzyme and inhibiting the other. Whether or not these enzymes are in different cellular or subcellular compartments is still an open question. Perhaps differential regulation by Ca^{2+} and cGMP provides functional compartmentalization of the phosphodiesterases and of cAMP.

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