THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 8, Issue of April 25, pp. 3757-3762, 1980
Printed in U.S.A.

Immunological Distinction between Guanosine 3':5'-Monophosphate-dependent and Adenosine 3':5'-Monophosphate-dependent Protein Kinases*

(Received for publication, September 24, 1979)

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A guanosine 3':5'-monophosphate (cGMP)-dependent protein kinase was purified from bovine lung using 8-(6-aminohexylamino)-cAMP-Sepharose. The activity of the purified enzyme was highly dependent on cGMP using histone f_2b as a substrate. The self-phosphorylation of the purified enzyme was strongly inhibited by cGMP and not significantly affected by cAMP. A precipitating antiserum prepared in rabbits against the cGMP-dependent protein kinase specifically inhibited the histone kinase activity and the self-phosphorylation of the purified cGMP-dependent protein kinase without affecting the cGMP binding site. This antiserum also specifically inhibited the phosphorylation of the endogenous substrate proteins by endogenous cGMP-dependent protein kinase in smooth muscle membranes, but did not cross-react detectably with catalytic subunit or regulatory subunit of type I or type II cAMP-dependent protein kinase. Conversely, antisera against the regulatory subunit of type I or type II cAMP-dependent protein kinase did not cross-react detectably with cGMP-dependent protein kinase. The substantial differences between the immunological properties of the cGMP-dependent and cAMP-dependent protein kinases suggest that these two enzymes have distinct physiological roles.

A cGMP-dependent protein kinase, initially discovered in several invertebrate tissues (1, 2), has been purified to homogeneity from bovine lung (3–5) and bovine heart (6). The enzyme has been shown to exist as a dimer composed of two identical subunits (M_r = 74,000 to 81,000) each of which appears to contain a cGMP binding site and catalytic activity. Whereas cAMP activates cAMP-dependent protein kinases by dissociating the tetrameric holoenzyme into regulatory and catalytic subunits, cGMP activates the cGMP-dependent protein kinase without such dissociation (3, 4). The cGMP-dependent protein kinase has been reported to be similar to cAMP-dependent protein kinases, especially the type II enzyme, with respect to several properties, including amino acid composition (7), various physical characteristics (7), ability to self-phosphorylate (5, 8), and even substrate specificity (7, 9, 10). This information led to the proposal that cAMP- and cGMP-dependent protein kinases are homologous proteins which evolved from a common ancestral protein (7, 11). However, specific endogenous substrates for the cGMP-dependent protein kinase have been described (12–15), and cGMP-dependent and cAMP-dependent protein kinases have been reported to exhibit differential substrate specificity using several synthetic peptides and histone H2B as substrates (16). In the present study, an immunological approach was chosen to compare the two types of protein kinase. A simplified procedure was developed for the purification of a protein kinase, the activity of which was highly dependent on cyclic GMP, and antibodies were made against this enzyme. Results of several experimental approaches demonstrated substantial antigenic differences between the cGMP-dependent and cAMP-dependent protein kinases both in the cyclic nucleotide-binding portion and the catalytic portion of the enzymes.

EXPERIMENTAL PROCEDURES

Materials—Cyclic AMP, cyclic GMP, ATP, 5'-AMP, histone f_2b, and DEAE-cellulose were purchased from Sigma, 3-isobutyl-1-methylxanthine from Aldrich, complete Freund's adjuvant from Calbiochem, CNBr-activated Sepharose 4B from Pharmacia, goat anti-rabbit IgG from Miles, and immunoplates from Hyland. Marker proteins (phosphorylase, BSA,[1] catalase, ovalbumin, aldolase, and chymotrypsinogen) were obtained from Boehringer. 8-(6-Aminohexylamino)cAMP (AHA-cAMP) was prepared as described (17) and coupled to CNBr-activated Sepharose 4B (18). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappel (19). 8-N_3-[^{32}P]cIMP was prepared as described (20). All other chemicals were reagent grade.

Purification of cGMP-dependent Protein Kinase—The enzyme was purified from bovine lung by a modification of published procedures (3, 4). All steps were performed at 4°C unless indicated otherwise. Bovine lung (1 kg), obtained from a local slaughterhouse, was cut into small pieces and homogenized in a Waring Blendor (four times at 15 s each) in 4 liters of 10 mM potassium phosphate (pH 6.8) containing 1 mM EDTA and 10 mM β-mercaptoethanol (PEM buffer). The homogenate was centrifuged at 10,000 × *g* for 30 min. The clear supernatant (3,800 ml) was added to a beaker containing 1 liter of solid DEAE-cellulose that had been equilibrated with PEM buffer. After gentle stirring for 30 min, the resin was filtered and washed once with 2 liters of PEM buffer. The DEAE-cellulose was then washed twice with 2 liters of PEM buffer containing 0.18 M NaCl. The two salt washes were combined and the protein was precipitated with $(NH_4)_2SO_4$ (400 g/liter). The precipitate was collected by centrifugation at 11,000 × *g* for 30 min, resuspended in about 100 ml of PEM buffer, and dialyzed overnight against three changes of the same buffer. The dialyzed solution was centrifuged at 20,000 × *g* for 30 min to remove any precipitated protein. The supernatant was then

* This work was supported by United States Public Health Service Grants DA-01627, MH-17387, and NS-08440, and by a grant from the McKnight Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: BSA, bovine serum albumin; AHA-cAMP, 8-(6-aminohexylamino)cAMP; 8-N_3-cIMP, 8-azido-inosine 3':5'-monophosphate; C, catalytic subunit of cAMP-dependent protein kinase; R-I, regulatory subunit of type I cAMP-dependent protein kinase; R-II, regulatory subunit of type II cAMP-dependent protein kinase; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N,N'N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G.

applied to a 3-ml column of AHA-cAMP-Sepharose 4B (2 μmol of AHA-cAMP/ml column) with a flow rate of about 15 ml/h. After the sample loading was completed, the AHA-cAMP-Sepharose was washed with 150 to 200 ml of PEM buffer containing 2 M NaCl at the same flow rate. The elution of the cGMP-dependent protein kinase from the AHA-cAMP-Sepharose column was then continued as described in detail in the legend of Fig. 1. The fraction containing the cGMP-dependent protein kinase was dialyzed for 24 h against several changes of PEM buffer. Traces of contamination in this fraction were removed by gel filtration. For this purpose the enzyme sample (4 ml) containing 2 to 4 mg of protein was applied to a Sephadex G-200 column (2.8 × 60 cm) that had been equilibrated with PEM buffer containing 50 mM NaCl. The column was eluted with the same buffer. Fractions containing cGMP-dependent protein kinase were pooled, concentrated, and dialyzed against PEM buffer. This enzyme, stored at a concentration of 0.3 to 0.5 mg/ml at 4°C, was used for all experiments.

Purification of Subunits of cAMP-dependent Protein Kinase—The catalytic subunit (C) was prepared from bovine heart by the method of Beavo *et al.* (21). The type I and type II regulatory subunits (R-I and R-II) were prepared by the method of Corbin *et al.* (22). The source of tissue was bovine lung in the case of R-I and bovine heart in the case of R-II.

Preparation of Antisera—Female New Zealand white rabbits (2 kg) were immunized with purified bovine lung cGMP-dependent protein kinase. Enzyme (0.1 mg/ml) dissolved in phosphate-buffered saline (0.9% NaCl solution) was emulsified with an equal volume of complete Freund's adjuvant. The emulsion (2 ml) was injected intradermally at multiple sites of the back of each rabbit. The animals were boostered with identical injections 11 and 22 days after the initial injection. Animals were bled from the ear vein 10 days after the last injection and at later times. A preimmune serum was obtained from the animals prior to immunization. All sera were stored at −20°C. Antisera against R-I and R-II were prepared by a similar procedure.[2]

Histone Kinase Assay—The activity of cGMP-dependent protein kinase was measured by a modification (20) of the procedure of Witt and Roskoski (23) using histone f_2b as substrate. The standard reaction mixture (final volume, 100 μl) contained 20 mM Tris-HCl (pH 7.1), 40 mM $MgCl_2$, 1 mM EGTA, 1 mM 3-isobutyl-1-methylxanthine, 10 mM β-mercaptoethanol, 100 μg of histone f_2b, 40 μM [γ-^{32}P]ATP (100 to 200 cpm/pmol), and the enzyme sample in the absence or presence of 1 μM cGMP. The reaction was initiated by the addition of the [γ-^{32}P]ATP, carried out for 4 min at 30°C, and terminated by the addition of 20 μl of 0.3 M EDTA. The samples were then analyzed for protein-bound [^{32}P]phosphate as described (20). When the effect of sera on the histone kinase activity was tested, the reaction mixture contained 10 μl of serum and was preincubated for 30 min at 0°C before the addition of the [γ-^{32}P]ATP.

The activity of the catalytic subunit of cAMP-dependent protein kinase and the effect of sera on this activity was measured similarly except for the use of 10 mM $MgCl_2$ and the absence of any cyclic nucleotide in the standard reaction mixture. It was noted that the presence of 10 μl of control serum slightly stimulated the activity of the cGMP-dependent protein kinase and slightly inhibited the activity of the catalytic subunit of cAMP-dependent protein kinase.

Self-phosphorylation of cGMP-dependent Protein Kinase—This reaction was assayed as described (5). The reaction mixture (final volume, 100 μl) contained 40 mM Tris-HCl (pH 7.3), 5 mM β-mercaptoethanol, 50 mM $MgCl_2$, and the enzyme sample in the absence or presence of 5 μM cGMP or 5 μM cAMP. After preincubation for 2 min at 30°C, the reaction was initiated by the addition of [γ-^{32}P]ATP, and terminated by the addition of 50 μl of "SDS-stop solution" (200 mM Tris-HCl (pH 6.7) containing 6% SDS, 6% β-mercaptoethanol, 15% glycerol, and a small amount of bromphenol blue). After boiling for 2 min, the entire sample was analyzed by SDS-polyacrylamide slab gel electrophoresis and autoradiography as previously described for the analysis of cAMP-dependent protein kinases (24, 25). When the effect of sera was tested, the reaction mixture contained 10 μl of serum, which had been diluted 1:10 in 10 mM Tris-HCl (pH 7.3), and was pre-preincubated for 30 min at 0°C prior to the 2-min preincubation at 30°C and addition of ATP.

Photoaffinity Labeling—Photoaffinity labeling experiments with 8-N_3-[^{32}P]cIMP were performed as described (20). The standard reaction mixture (final volume, 100 μl) contained 40 mM Tris-HCl (pH 7.3), 10 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 0.5 mM β-mercaptoethanol, 1 μM 8-N_3-[^{32}P]cIMP (8,000 to 10,000 cpm/pmol), the enzyme sample, 1 μM cGMP or 1 μM cAMP or no cyclic nucleotide.

Immunological Double Precipitation—A cytosol fraction was prepared from bovine pulmonary arteries as described (20). Type I and type II regulatory subunits (R-I and R-II) and the cGMP-dependent protein kinase present in 40 μl of cytosol (104 μg of protein) were radioactively labeled by 8-N_3-[^{32}P]cIMP using the standard conditions described above. 8-N_3-[^{32}P]cIMP was chosen as the photoaffinity label since it effectively labels both the cGMP-dependent protein kinase as well as the regulatory subunits of cAMP-dependent protein kinases (20). After preincubation and photolysis, 50 μl of antiserum or preimmune serum was added to 50 μl of the reaction mixture. (Antiserum and preimmune serum had been diluted 1:50 in phosphate-buffered saline containing 1 mg/ml of BSA.) The mixture was incubated for 30 min at 30°C and then overnight at 4°C. Twenty-five microliters of phosphate-buffered saline containing 150 μg of goat anti-rabbit IgG was then added to each sample and the samples were incubated for 15 min at 30°C and 2 h at 4°C. Precipitates were collected by centrifugation at 3,000 rpm for 30 min. The supernatants were removed and the pellets were washed twice with 0.5 ml of phosphate-buffered saline. The final pellet was dissolved in 100 μl of water and 50 μl of an SDS-stop solution and boiled for 2 min. The samples were then analyzed by SDS-polyacrylamide slab gel electrophoresis and autoradiography.

Endogenous Phosphorylation of Smooth Muscle Membranes—Bovine pulmonary arteries were obtained from a local slaughterhouse and the medial layer was dissected. A particulate fraction of the medial layer was prepared as described previously (12, 20) except that the homogenization buffer was 10 mM Tris-HCl (pH 7.4) containing 0.31 M sucrose, 0.3 mM EDTA and 10 mM β-mercaptoethanol. The standard reaction mixture for endogenous phosphorylation (final volume, 100 μl) contained 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, and 33 μg of protein from the particulate fraction in the absence or presence of 1 μM cGMP. Samples were preincubated for 1 min at 30°C. The reaction was started by the addition of [γ-^{32}P]ATP (60,000 to 80,000 cpm/pmol; final concentration, 3 μM), carried out for 20 s, and terminated by the addition of 50 μl of SDS-stop solution. Samples were further processed as described above in the procedure for self-phosphorylation. When the effect of sera was tested, samples were pre-preincubated with 10 μl of serum (diluted 1:20 in Tris-HCl, pH 7.4) for 30 min at 0°C prior to starting the preincubation reaction.

RESULTS AND DISCUSSION

The cGMP-dependent protein kinase from bovine lung was retained by 8-(6-aminohexylamino)-cAMP-Sepharose and eluted from it by cAMP (Fig. 1*A*). About 90% of the cGMP-dependent protein kinase retained was immediately eluted at 4°C by buffer containing 10 mM cAMP whereas the elution of the R-I and R-II regulatory subunits of cAMP-dependent protein kinase by this solution was only achieved at room temperature (Fig. 1*A*). It was noted that the complete elution of the R-I subunit required extensive washing of the affinity column at room temperature with buffer containing cAMP (data not shown). One kilogram of bovine lung usually yielded 3 to 4 mg of cGMP-dependent protein kinase eluted from the affinity column by cAMP. This is in close agreement with the highest yield reported (4). In contrast to other procedures (3, 6), no treatment other than dialysis was required for the removal of cyclic nucleotides in order to obtain a purified enzyme, the activity of which was highly dependent on cGMP. Freshly prepared enzyme had a specific activity (micromoles of ^{32}P transferred to histone f_2b per min and mg of protein) of 0.2 in the absence and of 1.3 in the presence of 1 μM cGMP when measured under standard conditions. Although the cGMP-dependent protein kinase eluted from the affinity column was of high purity, it did contain minor contaminants including traces of the R-I and R-II regulatory subunit (Fig. 1*A*). These contaminants could be removed by gel filtration using Sephadex G-200, and this enzyme of apparent homogeneity (Fig. 1*B*) was used for all subsequent studies.

The purified cGMP-dependent protein kinase was charac-

[2] S. M. Lohmann, U. Walter, and P. Greengard (1980), manuscript in preparation.

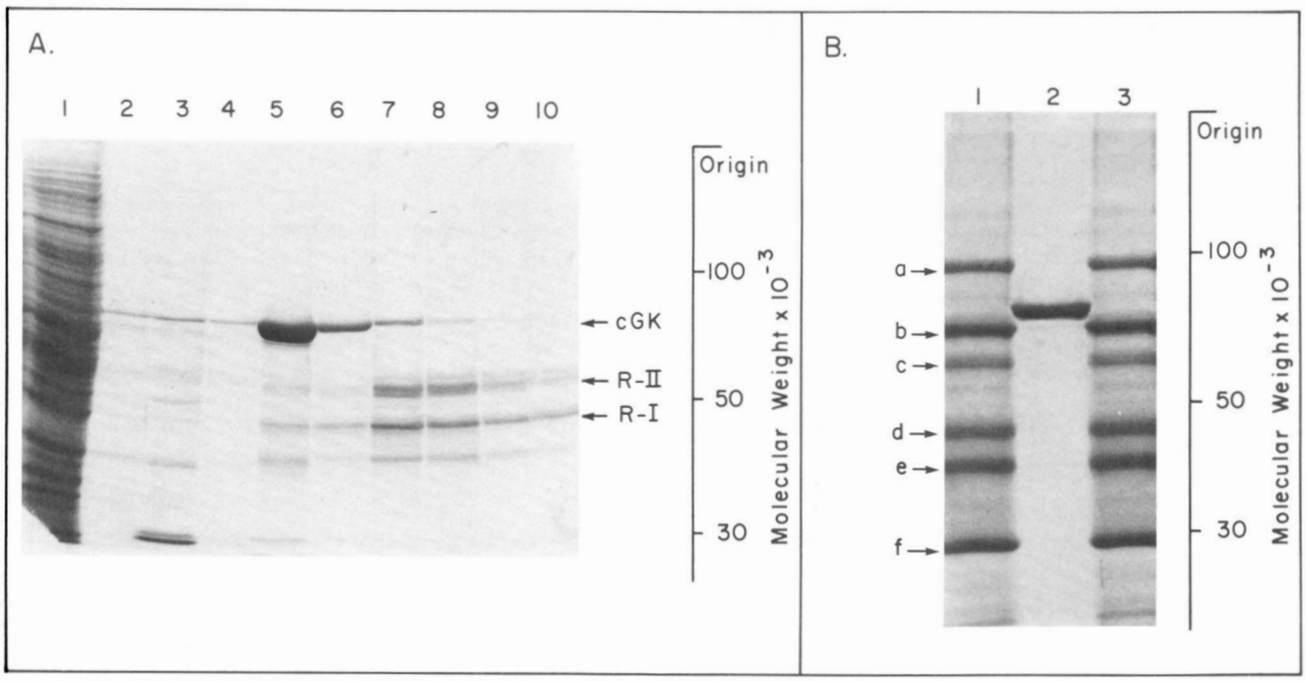

FIG. 1. **Elution of cGMP-dependent protein kinase from AHA-cAMP-Sepharose (*A*) and purified cGMP-dependent protein kinase (*B*), as illustrated by SDS-polyacrylamide slab gel electrophoresis.** *Panel A*, 50 μl of the fraction loaded onto the AHA-cAMP-sepharose (*Lane 1*) and of the various fractions eluted from it (*Lanes 2* to *10*) were analyzed for protein by SDS-polyacrylamide slab gel electrophoresis. After the AHA-cAMP-Sepharose column was loaded with the sample and then washed with PEM buffer containing 2 M NaCl (see "Experimental Procedures"), the column was eluted using flow rates of about 50 ml/h. The fractions analyzed were obtained by successively eluting the affinity column with 5-ml aliquots of PEM buffer (*Lane 2*), PEM buffer containing 10 mM 5'-AMP (*Lane 3*), PEM buffer (*Lane 4*), and PEM buffer containing 10 mM cAMP (*Lanes 5* to *10*). The elutions by PEM buffer containing 10 mM cAMP were performed in half-hour intervals at 4°C (*Lanes 5* and *6*) and at room temperature (*Lanes 7* to *10*). All previous steps were carried out at 4°C. The *arrows* indicate the position of cGMP-dependent protein kinase (*cGK*) and of the R-I and R-II regulatory subunits (*R-I* and *R-II*) of cAMP-dependent protein kinase. *Panel B*, ten micrograms of cGMP-dependent protein kinase that had been purified by affinity chromatography and subsequent gel filtration on Sephadex G-200 was analyzed by SDS-polyacrylamide slab gel electrophoresis (*Lane 2*). Protein markers of 7 μg each were also analyzed (*Lanes 1* and *3*): phosphorylase (*a*), BSA (*b*), catalase (*c*), ovalbumin (*d*), aldolase (*e*), and chymotrypsinogen (*f*).

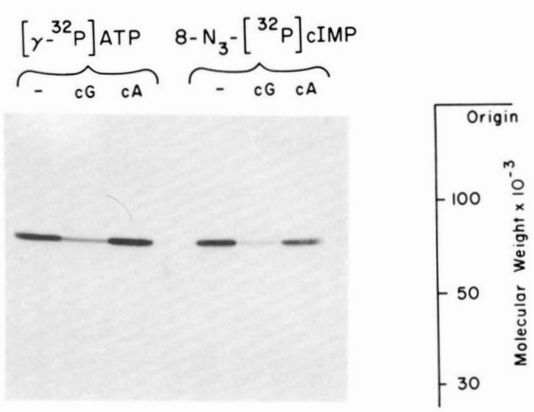

FIG. 2. **Autoradiograph showing the self-phosphorylation and photoaffinity labeling of purified cGMP-dependent protein kinase.** The enzyme (0.28 μg) was analyzed for the incorporation of phosphate from [γ-^{32}P]ATP under standard conditions in the absence or presence of cGMP (*cG*) or cAMP (*cA*). The enzyme (0.28 μg) was also analyzed for the photoactivated incorporation of 8-N$_3$-[^{32}P]cIMP under standard conditions in the absence or presence of cGMP (*cG*) or cAMP (*cA*).

terized by self-phosphorylation and by photoaffinity labeling. In agreement with DeJonge and Rosen (5), self-phosphorylation of the enzyme was observed in the absence of any cyclic nucleotide and was inhibited by the presence of cGMP (Fig. 2). In contrast to the results obtained by DeJonge and Rosen (5), cAMP did not stimulate the self-phosphorylation of our preparation of cGMP-dependent protein kinase (Fig. 2). In many experiments we even observed that cAMP partially mimicked the inhibitory effect of cGMP. It is possible that the difference in the results with respect to the effect of cAMP on self-phosphorylation could be due to the presence of some cGMP in the enzyme prepared by DeJonge and Rosen. The purified enzyme could also be labeled by the photoaffinity analog 8-N$_3$-[^{32}P]cIMP. This was prevented by 1 μM cGMP but inhibited to only a small extent by 1 μM cAMP (Fig. 2).

Injection of purified cGMP-dependent protein kinase into two different rabbits produced antisera in both animals, one of which was characterized in some detail. The antiserum against cGMP-dependent protein kinase contained precipitating antibodies which precipitated the purified cGMP-dependent protein kinase, but did not detectably precipitate the purified catalytic subunit or the R-I or R-II regulatory subunits from cAMP-dependent protein kinase (Fig. 3).

Using an immunological double precipitation technique, the antiserum against cGMP-dependent protein kinase specifically precipitated the 8-N$_3$-[^{32}P]cIMP-labeled cGMP-dependent protein kinase of bovine pulmonary artery cytosol without detectably precipitating the 8-N$_3$-[^{32}P]cIMP-labeled R-I and R-II regulatory subunit (Fig. 4). In contrast, antisera specific for either the R-I or the R-II regulatory subunit[2] precipitated the R-I or R-II regulatory subunit, respectively, but not the cGMP-dependent protein kinase of bovine pulmonary artery cytosol (Fig. 4).

Using histone f$_2$b as substrate, the antiserum inhibited both the basal and cGMP-dependent activity of purified cGMP-dependent protein kinase, and this inhibition was specific and dependent on the concentration of the antiserum (Fig. 5). The

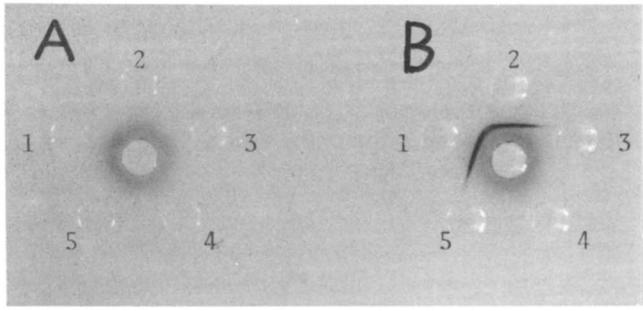

FIG. 3. **Ouchterlony double diffusion precipitin analysis of preimmune serum (*A*) and antiserum (*B*) prepared against cGMP-dependent protein kinase.** The immunoplates were incubated for 16 h in a moist chamber at room temperature, and then washed with saline for 24 h at 4°C. The plates were subsequently fixed and stained with a solution containing 10% acetic acid, 10% propanol, and 0.025% Coomassie blue, destained with 10% acetic acid and then photographed. The central wells contained 7.5 μl of preimmune serum (*A*) or antiserum (*B*). The outer wells contained 1.5 μg of purified cGMP-dependent protein kinase (*1* and *2*), or 1.5 μg of purified R-II regulatory subunit (*3*), purified catalytic subunit (*4*), or purified R-I regulatory subunit (*5*) of cAMP-dependent protein kinase.

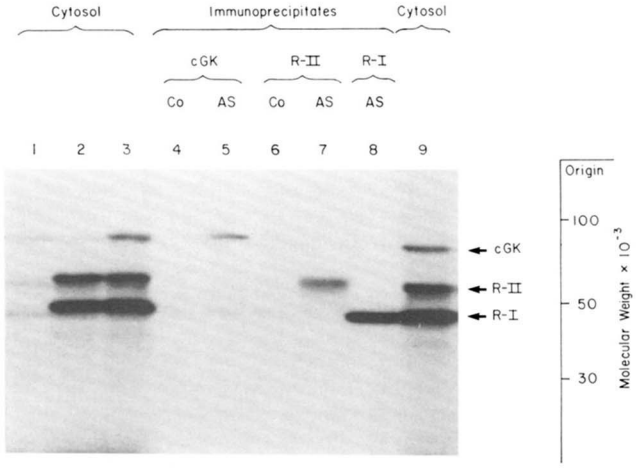

FIG. 4. **Autoradiograph showing the immunological double precipitation of cGMP-dependent protein kinase (*cGK*), type II regulatory subunit (*R-II*), and type I regulatory subunit (*R-I*) of cytosol from bovine pulmonary arteries.** 8-N_3-[^{32}P]cIMP incorporation was carried out under standard conditions in the absence of nonradioactive cyclic nucleotide. Immunoprecipitates (*Lanes 4* to *8*) were obtained and analyzed by the precipitation procedure described under "Experimental Procedures" using preimmune serum (*Co*, *Lane 4*) and antiserum (*AS*, *Lane 5*) against the cGMP-dependent protein kinase, preimmune serum (*Co*, *Lane 6*) and antiserum (*AS*, *Lane 7*) against the type II regulatory subunit (*R-II*), and antiserum (*AS*, *Lane 8*) against the type I regulatory subunit (*R-I*). Also shown are samples of photoaffinity-labeled cytosol (*Lanes 1* to *3* and *9*) identical with the original samples from which immunoprecipitates were made. In this case, the photoaffinity labeling was carried out under standard conditions in the absence of nonradioactive cyclic nucleotide (*Lanes 3* and *9*), in the presence of 1 μM cGMP (*Lane 2*), and in the presence of 20 μM cAMP (*Lane 1*) in order to demonstrate the labeling specificity of the cGMP-dependent protein kinase (*cGK*) and of the type I (*R-I*) and type II (*R-II*) regulatory subunits. The positions of these proteins are indicated.

antiserum had no effect on the activity of the purified catalytic subunit of cAMP-dependent protein kinase (Fig. 5), nor did it have an effect on the ability of cGMP-dependent protein kinase to bind [^{3}H]cGMP or to become labeled by 8-N_3-[^{32}P]cIMP (data not shown).

The antiserum inhibited the self-phosphorylation of purified cGMP-dependent protein kinase (Fig. 6). It did not change the effects of cGMP or cAMP on this reaction, which is consistent with our finding that cAMP and cGMP are able to bind to the enzyme in the presence of antiserum. When various dilutions of the antiserum were tested for their inhibitory effect on self-phosphorylation of the cGMP-dependent

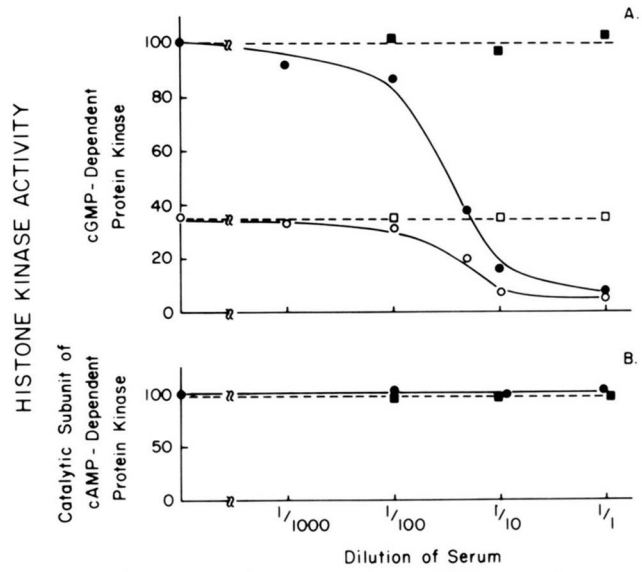

FIG. 5. **Effects of antiserum against cGMP-dependent protein kinase on the activity of purified cGMP-dependent protein kinase (*A*) and of purified catalytic subunit of cAMP-dependent protein kinase (*B*).** The histone kinase activity of 58 ng of purified cGMP-dependent protein kinase was assayed under standard conditions without (*open symbols*) or with (*closed symbols*) cGMP in the presence of various dilutions of preimmune serum (■- - -■, □- - -□) or antiserum (●——●, ○——○). The histone kinase activity of 58 ng of purified catalytic subunit was assayed under standard conditions in the presence of various dilutions of preimmune serum (■- - -■) or antiserum (●——●). All samples contained 10 μl of serum diluted as indicated by the serum of a control rabbit. The results are expressed as percentage of the activity measured in the presence of 10 μl of control serum. The endogenous histone kinase activity of the sera was less than 3% of the activity of both enzymes studied and was subtracted as blank.

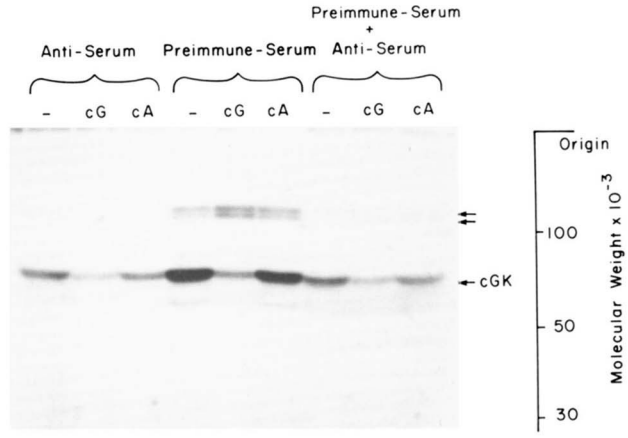

FIG. 6. **Autoradiograph showing the effect of antiserum against cGMP-dependent protein kinase on the self-phosphorylation of this enzyme.** The self-phosphorylation of purified cGMP-dependent protein kinase (58 ng) was assayed under standard conditions in the absence or presence of cGMP (*cG*) or cAMP (*cA*). The incubations were carried out in the presence of 10 μl of antiserum diluted 1:10, preimmune serum diluted 1:10, or both. The *arrows* indicate the position of the cGMP-dependent protein kinase (*cGK*) and the positions of two phosphorylated proteins present in the preimmune serum (*two upper arrows*).

protein kinase, a dilution curve similar to that shown in Fig. 5 was obtained (data not shown). However, it was frequently observed with the purified protein kinase that the inhibitory effect of the antiserum on self-phosphorylation was slightly less than its inhibitory effect on the histone kinase activity.

It was also noted that two proteins present in preimmune serum were phosphorylated during the assay of self-phosphorylation (Fig. 6, two upper arrows). Several lines of evidence suggested that these two proteins were phosphorylated by the added cGMP-dependent protein kinase and that the phosphorylation of similar proteins in the antiserum was prevented by the antibodies against the cGMP-dependent protein kinase: (*a*) these two proteins were not present in the preparation of purified cGMP-dependent protein kinase (Fig. 2) and were not phosphorylated in the serum without added cGMP-dependent protein kinase (data not shown); (*b*) adding antiserum to the preimmune serum inhibited the phosphorylation of these two proteins (Fig. 6); (*c*) the phosphorylation of these two proteins of the preimmune serum was slightly stimulated by cGMP and less so by cAMP (Fig. 6). (The relatively small stimulation by cGMP was most likely due to the limiting amounts of these proteins.)

The antiserum against the cGMP-dependent protein kinase from bovine lung was also tested for its effect on the phosphorylation of endogenous substrate proteins by endogenous cGMP-dependent protein kinase. A particulate fraction of smooth muscle was chosen for this experiment since smooth muscle is known to contain membrane-bound cGMP-dependent protein kinase and several membrane-bound substrates for the kinase (12, 14). In recent studies using rabbit aortic smooth muscle, evidence was obtained that these substrates for the endogenous cGMP-dependent protein kinase are integral proteins of the plasma membrane (26, 27). The cGMP-dependent protein kinase itself appears to exist in both a soluble form and as a peripheral plasma membrane protein, and results of peptide mapping indicate that the enzyme present in the particulate fraction is identical with that present in the soluble fraction (26, 27). Using a smooth muscle membrane fraction obtained from bovine pulmonary arteries, cGMP stimulated the endogenous phosphorylation of several substrate proteins designated G_0, G_1, G_2, and G_3 (Fig. 7). The antiserum against cGMP-dependent protein kinase inhibited the cGMP-stimulated phosphorylation of these four proteins while the preimmune serum did not (Fig. 7). The cGMP-independent phosphorylation of other proteins present in smooth muscle membranes was not affected either by the antiserum or by the preimmune serum. These two sera were also tested for possible effects on endogenous protein phosphorylation in a synaptic membrane fraction from bovine brain, a fraction known to support cAMP-dependent, but no detectable cGMP-dependent, phosphorylation of several substrate proteins (28, 29). Neither the preimmune serum nor the antiserum against cGMP-dependent protein kinase had any effect on the cAMP-dependent or -independent phosphorylation of proteins in this synaptic membrane fraction (data not shown). In other experiments the antiserum against cGMP-dependent protein kinase from bovine lung inhibited the endogenous cGMP-stimulated phosphorylation of substrate proteins in smooth muscle membranes of rabbit and rat aortae (data not shown). Antibodies against cGMP-dependent protein kinase were also able to inhibit selectively the cGMP-dependent protein phosphorylation in rat intestinal brush borders (H. R. de Jonge, Erasmus University, Rotterdam, personal communication). These results demonstrated that the antibodies of the antiserum studied not only recognized the soluble cGMP-dependent protein kinase from bovine lung but also the membrane-associated cGMP-dependent protein kinase from smooth muscle of several species, indicating that these enzymes share some antigenic determinants.

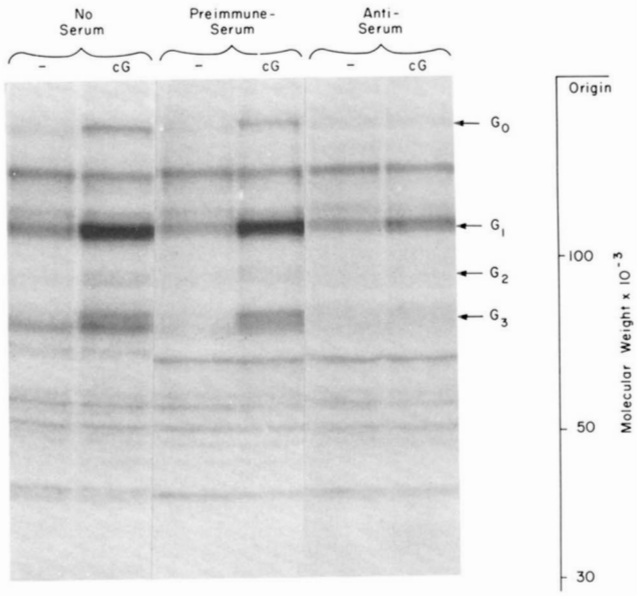

FIG. 7. **Autoradiograph showing the effect of antiserum against cGMP-dependent protein kinase on endogenous phosphorylation in smooth muscle membranes from bovine pulmonary arteries.** Endogenous phosphorylation was performed under standard conditions without or with cGMP (*cG*) using a particulate fraction (33 μg of protein) of smooth muscle. The incubations were carried out in the absence of any serum and in the presence of 10 μl of 1:20 diluted preimmune serum or antiserum. G_0, G_1, G_2, and G_3 identify the positions of those proteins whose phosphorylation was markedly stimulated by the presence of cGMP.

The antibodies studied appeared to bind at or near the catalytic site of the cGMP-dependent protein kinase without affecting the cGMP binding site. In contrast, immunization of guinea pigs with the type II cAMP-dependent protein kinase of bovine heart produced antibodies against only the regulatory subunit, which carries the cAMP binding site, but not against the catalytic subunit (30). The antibodies against the cGMP-dependent protein kinase did not detectably cross-react with, or inhibit, the activity of the catalytic subunit of cAMP-dependent protein kinase, demonstrating that these two proteins differ substantially in their antigenic determinants. A marked difference between the catalytic site of cGMP- and cAMP-dependent protein kinases has also been indicated by the fact that a heat-stable protein kinase modulator was found to inhibit the activity of cAMP-dependent protein kinase but not that of the cGMP-dependent protein kinase (3). In addition, the cGMP-dependent protein kinase appears to differ in its immunological properties from the two types of regulatory subunits of cAMP-dependent protein kinase. This is indicated by the observation that the antiserum against the cGMP-dependent protein kinase did not detectably cross-react with either the R-I or the R-II regulatory subunit (Figs. 3 and 4) and that antisera against the R-I or R-II regulatory subunit did not detectably cross-react with the cGMP-dependent protein kinase (Fig. 4). Although cGMP- and cAMP-dependent protein kinases have certain physical similarities (7), the immunological properties of these two enzymes do not support the concept (11) that cAMP- and cGMP-dependent protein kinases are homologous enzymes evolved from a common ancestral protein. However, a final answer to this question may require sequence analysis of the two protein kinases.

The substantial difference between cAMP-dependent and

cGMP-dependent protein kinases in their immunological properties, cyclic nucleotide specificity (1–4), and regulation by inhibitors (3), as well as the increasing evidence for differential substrate specificity of the two enzymes (12–16), appear to indicate that cGMP-dependent protein kinase has physiological roles which are distinct from those of cAMP-dependent protein kinase and which should be the subject of future investigations.

The specific properties of the antibody described in this report indicate that this antibody should be useful for studies of the precise cellular and subcellular localization of cGMP-dependent protein kinase in various tissues. An immunocytochemical approach to the localization of cyclic nucleotides and cAMP- and cGMP-dependent protein kinases has been discussed by Steiner *et al.* (31). The strong inhibitory effect of the antibody on the activity of cGMP-dependent protein kinase also indicates that this antibody may be useful in investigations of the physiological role of the cGMP-dependent protein kinase. Introduction of the antibody into a system with a defined physiological response to cGMP should result in inhibition of those events mediated by cGMP-dependent protein kinase.

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