A specific cGMP binding protein from rat lung has been partially purified and characterized. The binding protein is clearly distinct from the cGMP-dependent protein kinase as determined by several of its characteristics such as chromatographic behavior and spatial requirements for cGMP binding. The protein has a sedimentation coefficient of 7.8 S, a Stokes radius of 5.5 nm, and an estimated $M_r$ of 177,000.

A cGMP phosphodiesterase activity co-purifies with the binding protein through several steps (ion exchange chromatography, gel filtration, and sucrose density gradient centrifugation) and the possibility exists that these two activities may be functions of the same protein.

Several lines of evidence indicate that the cGMP binding site is distinct from the phosphodiesterase catalytic site. First, 1-methyl-3-isobutylxanthine is a competitive inhibitor of cGMP for phosphodiesterase activity, while under similar conditions this compound increases cGMP binding. Other phosphodiesterase inhibitors also increase cGMP binding and these effects are not due to substrate protection. Second, the cGMP analogue 2′-O-monomobutryl cGMP is an effective inhibitor of cGMP phosphodiesterase activity, but at the same concentration this compound has no effect on cGMP binding. Third, heat or trypsin treatment of the protein causes loss of cGMP phosphodiesterase activity, while both treatments increase cGMP binding.

Stimulation of protein phosphorylation is a major mechanism by which cyclic nucleotides exert their effects in eukaryotic cells (1-3). The cyclic AMP-dependent protein kinases (cAMP kinases) and cyclic GMP-dependent protein kinases (cGMP kinases) have been purified and characterized from many cell types. Many similarities between the two cyclic nucleotide-dependent protein kinases have been found (4, 5), particularly in substrate specificity, suggesting that they are homologous proteins. However, it is generally thought that the cGMP kinase has unique protein substrates and a specific role in mammalian tissues (6).

Escherichia coli contains a cAMP binding protein which is distinct from cAMP kinase (7), thus providing one instance in which the effects of cyclic nucleotides are not mediated by protein kinase. Several binding proteins for cAMP (8-10) and cGMP (6, 11-13) have been described in mammalian tissues. These proteins appear to be distinct from cAMP and cGMP kinases and from fragments derived from them, although their roles in mediating the actions of cyclic nucleotides are unknown.

Two distinct cGMP binding proteins in mammalian tissues have previously been resolved using DEAE-cellulose chromatography (13). One of these was identified as the cGMP kinase, while the other was not associated with cGMP-stimulated protein kinase activity. Both binding proteins bound cGMP with high specificity. In this report the cGMP binding protein which lacks cGMP kinase activity has been partially purified from rat lung and characterized with respect to some of its biochemical properties. A cGMP binding protein having some similar properties has recently been reported in rat platelets (14). The possible role of this binding protein in mediating some of the actions of cGMP in animal cells is discussed.

**EXPERIMENTAL PROCEDURES**

**Cyclic GMP Binding Assay**—Unless otherwise noted, cGMP binding activity was measured in a 100-μl volume containing 10 mM sodium phosphate (pH 7.0), 1 mM EDTA, 12 mM 2-mercaptoethanol, 0.5 μM [3H]cGMP (600,000 cpm), ± 0.2 mM 1-methyl-3-isobutylxanthine. Binding protein was added and the tubes were incubated at 0-4°C for 45 min. The mixture then was filtered on Millipore filters, and the filters were dried at 150°C and then counted in a toluene-based scintillant. Cyclic AMP (1 μM) was sometimes included in the binding assay in order to reduce nonspecific binding.

**Measurement of cGMP Kinase and Phosphodiesterase Activities**—Cyclic GMP kinase was measured as described previously (15) in the presence or absence of 0.5 μM cGMP unless otherwise indicated. Assays were conducted at 30°C for 10 min. cGMP phosphodiesterase activity was measured according to the method of Wells et al. (16). Enzyme was added to a final volume of 150 μl containing 50 mM Tris-Cl (pH 7.5), 25 mM MgCl₂, 0.33 mg/ml of "metal-free" bovine serum albumin, and 1.0 μM [3H]cGMP unless otherwise indicated. After 5 to 15 min at 30°C the reaction was stopped by the addition of 20 μl of a mixture containing 10 mM cGMP, 50 mM EDTA, 30 mM theophylline, and 100 mM Tris, pH 7.5. Two hundred microliters of *Crotalus atrox* snake venom 5' nucleotidase was added for another 10 min at 30°C and the reaction was terminated by the addition of 1 ml of 15 mM EDTA, 0.1 mM adenosine, and 0.1 mM γ-aminonucleoside. The mixture was chromatographed over quaternary amine (QAE)-Sephadex and the effluent was counted in a toluene-based scintillant (17). Protein was measured by a modification (18) of the method of Lowry et al. (19).

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cGMP Binding Protein

Other Methods—Sucrose density gradient centrifugation was performed by the method of Martin and Ames (20). Standards used for the calculation of the sedimentation coefficient were catalase (11.2 S), phosphorylase b (8.2 S), and hemoglobin (4.6 S). The Sepharose 6B column used for determination of the Stokes radius of the binding protein was calibrated with blue dextran, apoferritin (5.94 nm), catalase (5.2 nm), bovine serum albumin (3.5 nm), ovalbumin (2.7 nm), type II collagen (0.95 nm) subunit of protein kinase (5.4 nm), and [3H]H2O. The column was equilibrated with 20 mM sodium phosphate (pH 7.0), 2 mM EDTA, 25 mM 2-mercaptoethanol. In some cases 0.2 mM methylisobutylxanthine and 25 mM MgCl2 were also included in the buffer. For the separation of possible products of cGMP metabolism, test material made 5% in trichloroacetic acid was chromatographed on Dowex 50 H+ (17).

Materials—[3H]cGMP (21 Ci/mmol) and [γ-32P]ATP were from Amersham/Searle. DE11 was purchased from Whatman Co. Hemoglobin was from Nutritional Biochemicals Corp. Type II A histone, 8-bromo-cAMP, 6-bromo-cGMP, 2'-O-monobutyryl cGMP, 2'-O-succinyl cGMP, 2'-O-succinyl cGMP, 2-N-monobutyryl cGMP, cGMP, CAMP, trypsin, soybean trypsin inhibitor, bovine serum albumin, ovalbumin, catalase, methylisobutylxanthine, and C. atrox snake venom 5'-nucleotidase were from Sigma Co. 2'-N'-2'-Dibutyryl cGMP and 2'-3'-cGMP were from Boehringer Mannheim. Apoferritin was from Worthington. Bovine serum albumin was treated with ethylene glycol bis(β-aminomethyl ether)N,N'-tetraacetic acid and EDTA prior to use to provide “metal-free” bovine serum albumin according to the method of Wells and Garbers (21).

RESULTS

Purification—Male Sprague-Dawley rats (300 g) were used. Animals were killed by decapitation and the lungs were quickly removed and placed on ice. All subsequent steps were performed at 0-4°C. Approximately 140 g of rat lung were homogenized in 5 volumes (5 ml/g of tissue) of 20 mM sodium phosphate, 2 mM EDTA, 25 mM 2-mercaptoethanol, pH 7.0 (PEM buffer). The homogenate was centrifuged at 12,000 x g for 30 min and the supernatant was applied to a DEAE-cellulose column (2.6 x 30 cm) equilibrated with the same buffer. The column was washed with 5 volumes of buffer and eluted with a 4-liter linear gradient of NaCl from 0 to 0.4 M. Fractions of 25 ml (160 fractions) were collected and assayed for cGMP binding activity. The column profile is shown in Fig. 1A. Under these conditions, no cGMP binding activity emerged in the flow-through volume.

Two peaks of cGMP binding activity eluting at 70 and 120 mM NaCl, respectively, were resolved. At a cGMP concentration of 0.5 μM, cGMP binding activity in the first peak was markedly stimulated by 0.2 mM methylisobutylxanthine (Fig. 1A) and a cGMP phosphodiesterase activity (Fig. 1B) co-eluted with the binding activity. This cGMP binding protein-phosphodiesterase was clearly separated from the peak of cGMP kinase (Fig. 1B), which eluted with the second peak of cGMP binding. A small peak of protein kinase activity eluted on the front portion of the first cGMP binding peak but this activity was unaffected by cyclic nucleotides. The cGMP binding activities of the fractions of the first and second DEAE-cellulose peaks could be pooled separately and rechromatographed in their original positions (not shown).

The two cGMP binding peaks were pooled separately, and the binding activities were precipitated with 60% ammonium sulfate. After resuspension of the pellets and dialysis for 12 h against 40 volumes of PEM buffer, the concentrated protein solutions were centrifuged at 12,000 x g for 30 min and portions of the supernatants were chromatographed separately on a Sepharose 6B column (1.5 x 90 cm) equilibrated with PEM buffer. The column was eluted and fractions (approximately 2.6 ml) were collected and assayed for cGMP binding in the presence and absence of methylisobutylxanthine and for cGMP phosphodiesterase activities. Upon Sepharose 6B chromatography the cGMP phosphodiesterase and the cGMP binding which is increased by 1-methyl-3-isobutylxanthine again co-eluted (Fig. 2), and no cGMP kinase activity was associated with this peak (not shown). The second peak of cGMP binding from the DEAE-cellulose column eluted at a higher elution volume on Sepharose 6B than the first peak and co-eluted with protein kinase activity which was stimulated 2- to 3-fold by cGMP (not shown). At 0.5 μM [3H]cGMP the specific binding activity of the partially purified cGMP binding protein-phosphodiesterase was 9.1 pmol of CGMP bound/mg of protein in the presence of methylisobutyloxanthine. This fraction was used for further studies of the protein.

Properties—When histone was used as a substrate no cGMP nor CAMP-stimulated protein kinase was associated with the cGMP binding protein. The cGMP binding protein-phosphodiesterase sedimented at 7.8 S in sucrose density gradients (not shown) and had a Stokes radius of 5.5 nm as determined on a Sepharose 6B column. These parameters were not altered in the presence of 0.2 mM methylisobutylxanthine or 25 mM MgCl2. The cGMP binding protein-phosphodiesterase was estimated to have a Mr = 177,000 using the method of Seigel and Monty (22). The calculated frictional ratio of the protein was estimated to be 1.54, indicating marked asymmetry in the conformation.
The fact that the native cGMP binding protein-phosphodiesterase was larger than the cGMP kinase \( (M_r = 165,000) \) suggested that it was not a product of proteolysis of the cGMP kinase. Furthermore, efforts to convert the cGMP binding protein-phosphodiesterase to the cGMP kinase by proteolytic digestion were unsuccessful. Using several combinations of assay conditions for cGMP kinase, we have not found any cGMP kinase activity in the cGMP binding protein-phosphodiesterase fraction. Thus, all evidence suggested that the cGMP binding protein-phosphodiesterase is a unique cGMP binding protein not related to the cGMP kinase.

The concentration of cGMP which results in half-saturation of the partially purified protein in the presence of methylisobutylxanthine was approximately 0.5 to 1 \( \mu M \) (Fig. 3). The methylisobutylxanthine appeared to increase the affinity of the binding protein for cGMP but under our conditions binding in the absence of methylisobutylxanthine is not saturated, so it is not possible to state whether methylisobutylxanthine increases the total number of available binding sites. The binding data in Fig. 3 were obtained using the same protein concentration in the presence and absence of methylisobutylxanthine. Whether the cGMP binding kinetics are affected by protein concentration and other factors, which is the case for the cAMP kinase, is not known. There appeared to be two components of cGMP binding in this preparation as seen in Fig. 3, one with high affinity and another of a lower affinity. Until a more highly purified preparation of binding protein is obtained, however, it is not possible to clearly determine the binding characteristics and the mechanism of methylisobutylxanthine stimulation. Other phosphodiesterase inhibitors, such as papaverine, which is not a methylxanthine derivative, were also effective in stimulating cGMP binding. Half-maximal stimulation of binding by methylisobutylxanthine was observed at approximately 0.02 to 0.05 \( \mu M \). The order of potency for the stimulation of binding by phosphodiesterase inhibitors was methylisobutylxanthine = papaverine > theophylline > caffeine. We have found that this is the same order of potency for inhibition of cGMP hydrolysis by the phosphodiesterase which accompanies the binding activity. The concentration at which methylisobutylxanthine affected phosphodiesterase activity was lower than that which increased the cGMP binding, but differences in experimental conditions for the two assays may explain this observation.

The stimulation of \(^{[3}H\)cGMP binding by methylisobutylxanthine was not due to preservation of the ligand due to inhibition of phosphodiesterase activity in the binding assay. Efforts to detect phosphodiesterase activity under the conditions used for the binding assay (1 \( \mu M \) EDTA, 0-4°C) in the absence and presence of methylisobutylxanthine was unsuccessful. No stimulation of cGMP binding by methylisobutylxanthine was observed for the cGMP kinase fraction. The labeled material bound to the Millipore filter in the binding studies was identified as \(^{[3}H\)cGMP. Filter papers containing cGMP binding protein-phosphodiesterase with \(^{3}H\) label bound were extracted with deionized water at 0-4°C for 1 h. The extracts were made 5% trichloroacetic acid, 0.1 \( \mu M \) cGMP, and 0.1 \( \mu M \) GMP and samples were immediately applied to Dowex 50-H+ columns (17). The columns were eluted with 0.1 N HCl and absorbance at 260 nm was correlated with elution of radioactivity. All radioactivity was associated with the cGMP peak and none correlated with the GMP.

Magnesium (5 \( \mu M \)) also increased the binding of cGMP by the cGMP binding protein-phosphodiesterase 5-fold in the absence of methylisobutylxanthine. In the presence of 0.2 \( \mu M \) methylisobutylxanthine, 5 \( \mu M \) magnesium produced a 2-fold increase in cGMP binding. This effect occurred at concentrations of magnesium as low as 0.05 \( \mu M \) and was reversed by the addition of EDTA.

**Specificity**—At a ratio of nucleotide or nucleoside/cGMP of 20:1, there was no effect on \(^{[3}H\)cGMP binding by various nonradioactive adenine and guanine nucleosides and nucleotides either in the presence or absence of methylisobutylxanthine (not shown). Compounds which were shown to have no effect on either activity included guanosine, 5'-GMP, GDP,
GTP, 8-bromo-cGMP, adenosine, 5'-AMP, ADP, ATP, 3':5'-cAMP, dibutyryl cAMP, 8-oxo-cAMP, 3':5'-cUMP and 5'-UMP. As shown in Table I, cIMP significantly interfered with cGMP binding but cIMP inhibited the phosphodiesterase as well. Substitution of a succinyl group at the 2'-hydroxyl position diminished the ability of cIMP to inhibit the binding and the phosphodiesterase. No breakdown of [3H]cGMP was observed during standard binding assays.

However, differences in the specificity for the cGMP binding and the cGMP phosphodiesterase could be demonstrated using cGMP derivatives. As can be seen in Table I there was a marked inhibitory effect of 2'-O-monobutyryl cGMP and 2':3'-cGMP on phosphodiesterase activity, while there was no apparent effect of these derivatives on cGMP binding activity. Replacing the butyryl group with a succinyl group decreased the inhibition of the phosphodiesterase. The 2'-O-succinyl cGMP tyrosine methyl ester was a good inhibitor of both cGMP binding and cGMP phosphodiesterase.

Since the cGMP phosphodiesterase and binding assays were done under slightly different conditions in the experiment of Table I, it was decided to examine the effects of the most selective compound, 2'-O-monobutyryl cGMP, on the two activities under identical conditions. It can be seen in Table II that, at a 20:1 ratio of 2'-O-monobutyryl cGMP/cGMP, the hydrolysis of cGMP was markedly inhibited (72%) by the monobutyryl cGMP derivative, whereas the cGMP binding was unaffected. Sodium butyrate had no effect on the phosphodiesterase activity. A maximum of 35% of the substrate was hydrolyzed in the control sample in this assay. The 2'-O-monobutyryl cGMP had no effect in the 5'-nucleotidase reaction. Noncompetitive effects of cGMP derivatives on phosphodiesterase activity could not be ruled out in these studies. Yet, the data of Tables I and II provide strong evidence that there is a cGMP phosphodiesterase site with spatial requirements which differ from those occurring in the cGMP binding site.

Distinctive differences in the binding of cGMP by the cGMP binding protein-phosphodiesterase and the cGMP kinase also exist since low concentrations of 8-bromo-cAMP and 8-bromo-cGMP readily competed with [3H]cGMP for the cGMP kinase binding but not for the cGMP binding protein-phosphodiesterase binding (Fig. 4). These results suggested that the spatial requirements in the cGMP binding site of the cGMP binding protein-phosphodiesterase are very different from those in the cGMP site on the cGMP kinase.

**Relationship of the cGMP Binding Protein to cGMP Phosphodiesterase.**—In addition to their similar behavior on DEAE-cellulose and Sepharose 6B, the methylisobutylxan...
thine-stimulated cGMP binding and cGMP phosphodiesterase activities co-migrated on sucrose density gradients and non-denaturing polyacrylamide gel electrophoresis (not shown). These results suggested that the cGMP binding protein and the cGMP phosphodiesterase are either the same protein or very closely related proteins. The phosphodiesterase activity present in the binding protein preparation preferred cGMP to cAMP; at 1 μM nucleotide concentrations cGMP was hydrolyzed at an approximately 50 times greater rate. Half-maximal hydrolysis of cGMP occurred at 4 μM and the inhibition of the cGMP phosphodiesterase activity by methylisobutyloxanthine showed competitive kinetics with cGMP. In the presence of 100 μM calcium chloride, calmodulin had no effect on cGMP phosphodiesterase or on cGMP binding activity.

Effects of Trypsin and Heat Treatment—Fig. 5 shows that trypsin treatment (20 μg/ml at 30°C) increased the cGMP binding activity. After trypsin treatment the binding of cGMP by the protein was no longer affected by methylisobutyloxanthine. Magnesium also did not affect cGMP binding after trypsin treatment (not shown). The cGMP binding in the trypsinized sample at 30 min exceeded that observed with methylisobutyloxanthine in the native protein, suggesting that additional cGMP binding sites were available under these circumstances. The cGMP phosphodiesterase activity was destroyed in 5 min of trypsin treatment (not shown). The trypsinized cGMP binding protein-phosphodiesterase had a Stokes radius of 4.27 nm, a sedimentation coefficient of 6, a frictional ratio of 1.42, and an estimated Mr of 105,000. The trypsinized protein was more electronegative than the native cGMP binding protein-phosphodiesterase since it eluted at a higher salt concentration from DEAE-cellulose. The affinity of the trypsinized protein for cGMP was not significantly different from that observed with the native cGMP binding protein-phosphodiesterase in the presence of methylisobutyloxanthine (Fig. 3). The trypsinized cGMP binding protein-phosphodiesterase also appeared to retain its specificity for cGMP binding since cAMP, GMP, or 2′-O-monobutyryl cGMP did not affect the binding of [3H]cGMP.

Incubation of the cGMP binding protein-phosphodiesterase at 40°C for 2 to 5 min caused rapid loss of phosphodiesterase activity. The stimulation of [3H]cGMP binding by methylisobutyloxanthine was also destroyed by this treatment but [3H]cGMP binding in the absence of methylisobutyloxanthine was increased (not shown).

FIG. 5. Effects of trypsinization on cGMP binding. The binding protein was incubated with trypsin at a binding protein-phosphodiesterase/trypsin ratio of 100:1 at 30°C for the times indicated. Trypsinization was stopped by the addition of a 4-fold excess of soybean trypsin inhibitor. The samples were placed on ice and the cGMP binding assay was begun immediately.
contaminating protein of lower cGMP binding affinity. However, it is clear that rapid and specific cGMP binding by cGMP binding protein-phosphodiesterase occurs in the physiological range of cGMP concentration.

The mechanism of stimulation of cGMP binding by phosphodiesterase inhibitors is not known. However, all evidence suggests that the site to which cGMP binds in this protein is distinct from that which hydrolyzes the nucleotide: 1) competitive inhibitors of cGMP hydrolysis, such as methylisobutyxanthine, do not inhibit but, rather, enhance cGMP binding to this protein fraction; 2) heat or trypsin treatment destroys the phosphodiesterase activity but increases cGMP binding; 3) 2'-O-monobutyryl cGMP selectively inhibits the cGMP phosphodiesterase but has no effect on the cGMP binding.

The latter observation suggests that the cGMP binding site is more sensitive than the phosphodiesterase site to modification of the 2'-hydroxyl group. Studies of cGMP derivatives suggest the phosphodiesterase catalytic site has less specific spatial requirements than does the cGMP binding site. The stimulatory effects of methylisobutyxanthine, magnesium, and trypsin on cGMP binding suggested that these agents relieve an inhibitory component of the protein for cGMP binding. It is possible that these effects could be mimicked by endogenous factor(s).

It is clearly possible that the binding protein is identical to the cGMP phosphodiesterase which seems to co-purify with it. If so, the data indicate that there are two cGMP sites on the enzyme, a hydrolytic site and an allosteric site. In such a model methylisobutyxanthine binding at the hydrolytic site would increase the cGMP binding at the allosteric position. Binding of cGMP at the allosteric site may in turn affect phosphodiesterase activity in some manner. However, it is possible that co-purification is coincidental and the cGMP phosphodiesterase activity and the cGMP binding activity are really distinct proteins. Co-purification would be expected if the protein containing the binding activity were structurally very similar to the phosphodiesterase, with binding sites for cGMP and for phosphodiesterase inhibitors such as methylisobutyxanthine. Purification of the binding protein and characterization of its subunit composition and kinetic parameters is necessary before definitive statements regarding its separate identity and regulation of cGMP binding and hydrolysis can be made.

There is evidence that the methylxanthines have metabolic effects other than inhibition of phosphodiesterases (27-29). Whether any of these effects are mediated by the cGMP binding protein reported here remains to be determined. The specific effects of methylxanthines and magnesium on cGMP binding by binding protein-phosphodiesterase raises the possibility that cGMP binding may be modulated by other factors present in the cell. By increasing the amount of cGMP bound to a receptor in the cell the concentration of its "activated receptor" could be increased in the absence of any overall changes in the concentration of cGMP.

Although the role of the cGMP binding protein-phosphodiesterase is not known, its occurrence in cells such as sea urchin sperm and rat platelets where the cGMP kinase appears to be virtually absent suggests that it could be a cGMP receptor in these cell types. Furthermore, its apparent abundance in mammalian tissues, such as platelets (14) and lung, may indicate a functional role for the protein in these tissues as well. Whether the cGMP binding protein of rat small intestine, which has a sedimentation coefficient of 6 S (13), is derived from cGMP binding protein-phosphodiesterase remains to be determined.

Goldberg and Haddox have suggested that the energy of hydrolysis of cGMP (and other cyclic nucleotides also) could be coupled to the regulation of other cellular processes (6). The energy derived from the hydrolysis of cGMP could be coupled to the control of some cellular function. If this binding protein is a cGMP phosphodiesterase, it may provide a unique system for studying allosteric regulation of cyclic nucleotide hydrolysis. The cGMP phosphodiesterase activity in our study has many similarities with the cGMP phosphodiesterase purified from guinea pig lung by Kuo et al. (30). However, there are distinctive differences in the two activities, e.g. molecular weight and the effects of cobalt on activity.

There is much evidence to suggest that the cGMP kinase is a major receptor for cGMP in several tissues; however, all effects of cyclic nucleotides may not be mediated by protein kinases. It has been proposed that the cGMP kinase may not have as widespread a role in the control of cell function as the cAMP kinases (31). The earlier proposal was based on the limited distribution of the cGMP kinase and its limited catalytic ability. Our results presented here suggest that the effects of cGMP binding may be mediated not only by control of protein phosphorylation but by other mechanisms as well.

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