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A Simple, Sensitive Protein-binding Assay for Guanosine 3′:5′-Monophosphate

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ABSTRACT A protein-binding assay for guanosine 3′:5′-monophosphate, which employs an enzyme preparation from lobster tail muscle, is described. The binding constant for cyclic GMP was 2-10 nM for four different enzyme preparations. The method can be set up in several days and permits the detection of as little as 0.5-1 pmol of cyclic GMP. In some instances, it is possible to assay the nucleotide in urine or deproteinized extracts of tissue without purification. For other samples, a simple method for separating and purifying cyclic AMP and cyclic GMP is described.

Although guanosine 3′:5′-monophosphate (cyclic GMP) has been found in urine and many tissues (1–5), its physiological role remains to be established. Investigation in this area has been hampered by the relatively difficult and/or insensitive methods currently available for its assay, and the necessity of complicated purification procedures before assay (2, 3, 6). Assays for cyclic AMP, using preparations of cyclic AMP-binding protein from skeletal muscle (7) or adrenal (8), have recently been described. The cyclic AMP-binding protein is apparently a component of preparations that contain cyclic AMP-stimulated protein kinase activity. We have developed a similar binding assay for cyclic GMP using a protein fraction from lobster muscle that contains cyclic GMP-stimulated protein kinase activity (9). The method described permits the detection of as little as 1 pmol of cyclic GMP. Deproteinized extracts from some sources can be assayed directly, but samples from tissues in which cyclic GMP concentrations are relatively low require prior purification. A simple method for separation and purification of cyclic GMP and cyclic AMP is also described.

MATERIALS AND METHODS

Preparation of enzyme

The protein preparation used for binding of cyclic GMP was prepared from lobster tail muscle, through the dialyzed $(NH_4)_2SO_4$ step, by essentially the same procedures as described by Kuo and Greengard (9) for the preparation of a cyclic GMP-dependent protein kinase. Protein-kinase activity (9, 10) at this stage of purification was stimulated about 2.5-fold with 1 μM cyclic GMP and 2-fold with 1 μM cyclic AMP. The protein-kinase activity of the homogenate, and of the pH 4.9 supernatant fraction, was essentially unaltered by cyclic GMP or cyclic AMP. Attempts to further purify the dialyzed $(NH_4)_2SO_4$ fraction by chromatography on DEAE-cellulose, carboxymethyl-cellulose, phosphocellulose, or gel filtration with Sephadex G-100 resulted in large losses in both protein-kinase and cyclic GMP-binding activities. The dialyzed $(NH_4)_2SO_4$ fractions have been stored at −70°C for several months without significant loss of binding activity. With each thawing and refreezing of a preparation, however, about 5–10% of the binding activity is lost. For use in the assay, the dialyzed preparation (11–15 mg protein/ml) was divided into 1- to 2-ml portions, which were stored at −70°C.

Preparation of tissue samples

Male Wistar rats were anesthetized with ether and pieces of liver, lung, and cerebellum were rapidly removed, placed in liquid nitrogen, and subsequently homogenized in 5% trichloroacetic acid. After centrifugation, HCl was added to supernatant fractions to give a concentration of 0.05 N. Samples were extracted several times with ether, lyophilized, and dissolved in 50 mM Na acetate (pH 4.0). Aliquots were either assayed directly, or applied to 0.5 × 3-cm Dowex AG 1 × 8 formate (200–400 mesh) columns; then the column was washed with 10 ml of redistilled water. Cyclic AMP, along with 5′ AMP, was eluted with 10 ml of 2 N formic acid. Cyclic GMP, along with 5′ GMP and ADP, was next eluted with 14 ml of 4 N formic acid. Under these conditions, ATP was not eluted from the columns. Column eluates were lyophilized and dissolved in 50 mM Na acetate (pH 4.0) for assay of cyclic GMP and cyclic AMP content. When radioactive cyclic GMP and/or cyclic AMP were added to crude extracts before purification, we found that the purification method described resulted in complete separation and nearly quantitative recovery (80–95%) of each of the cyclic nucleotides. Samples of urine were assayed directly, or after purification on Dowex AG 1 × 8 formate.

The protein inhibitor of cyclic AMP-stimulated protein kinase (ref. 11 and D. A. Walsh, personal communication) was prepared through the dialyzed TCA-precipitated fraction, as described by Appleman *et al.* (12). Protein kinase activity of enzyme fractions was determined as described by Kuo and Greengard (9, 10). Protein was determined by the method of Lowry *et al.*, with bovine serum albumin as a standard (13). Cyclic AMP was assayed with the protein-binding assay described by Gilman (7), employing a protein-kinase preparation from bovine skeletal muscle.

[^{3}H]Cyclic GMP (4.45 Ci/mmol) and [^{3}H]cyclic AMP (16.3 Ci/mmol) were purchased from Schwarz BioResearch. [γ-^{32}P]ATP was prepared by the method of Post and Sen (14) and kindly donated by Drs. L. Shen and C. Villar-Palasi. Other reagents and nucleotides were obtained or made as described (15, 16). Cellulose ester filters (Cat. No. HAWP-02500) were

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purchased from Millipore Corporation. Values reported are means of duplicate incubations, except where noted.

RESULTS

Assay conditions

To a 10 × 75-mm disposable tube was added 5 μmol of Na acetate (pH 4.0), 6 or 10 pmol of [^{3}H]cyclic GMP, and cyclic GMP standards or unknowns. The tubes were placed in an ice bath and a dialyzed $(NH_4)_2SO_4$ fraction from lobster muscle (usually 130 or 260 μg of protein) was added to give a final volume of 0.1 ml. After incubation in an ice bath for 75 min, the samples were diluted to 1 ml with cold 20 mM potassium phosphate buffer (pH 6.2) and filtered (7). After they were washed with 10 ml of the same cold buffer, the filters were dissolved in 12 ml of a mixture containing 8.6 ml of toluene, 3.4 ml of ethylene glycol monomethyl ether, 43 mg of 2,5-diphenyloxazole (PPO), and 0.54 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP), and counted in a liquid scintillation spectrometer. Ethylene glycol monomethyl ether rather than ethylene glycol monoethyl ether (Cellosolve) (7) was used since filters were dissolved more readily and counting efficiency was increased (34 vs. 27%).

The amount of [^{3}H]cyclic GMP–protein complex recovered on a single membrane filter was directly proportional to the amount of protein added. Such proportionality was not observed when Na acetate (pH 4.0) was used instead of the phosphate buffer for dilution and washing. The amount of cyclic GMP bound was maximal after 75 min of incubation and was constant for at least 45 min thereafter. The binding constant under the conditions of the assay varied from 2 to 10 nM with four different enzyme preparations (Fig. 1). The specific activities of the enzyme preparations (pmol cyclic GMP bound/mg protein) varied from 0.24 to 0.77. At saturating concentrations of cyclic GMP, and at concentrations near the binding constant, binding was maximal with Na acetate buffer, pH 4.0 (Fig. 2). Other buffers in this pH range were not tested.

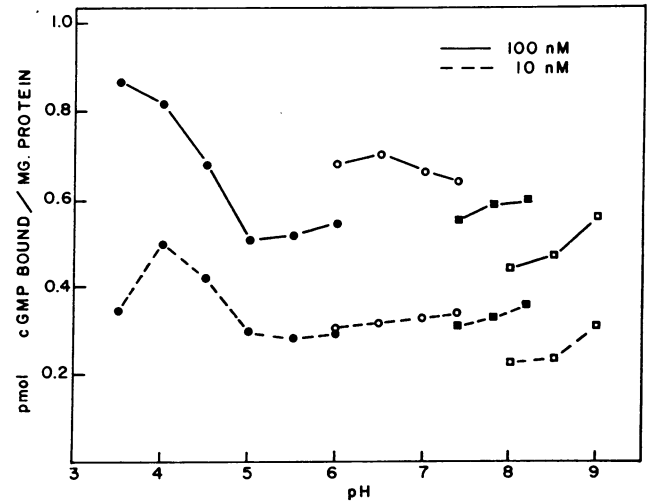

FIG. 2. Effect of pH on cyclic GMP binding. Incubations were performed with either 10 pmol (100 nM) (——) or 1 pmol (10 nM) (- - -) of [^{3}H]cyclic GMP and 260 μg of protein in 0.1 ml at the pH indicated. Buffers used were Na acetate (●), potassium phosphate (○), Tris–chloride (■), and Na glycinate (□).

With the enzyme preparation used in the experiments shown in Fig. 3, saturation of binding was observed at 60 nM cyclic GMP (6 pmol/0.1 ml, or 3 pmol/0.05 ml). Addition of known amounts of unlabeled cyclic GMP decreased the amount of [^{3}H]cyclic GMP bound in a manner such that linear and nearly theoretical curves were obtained when data were plotted logarithmically. From such standard curves, the cyclic GMP content of unknown samples was determined.

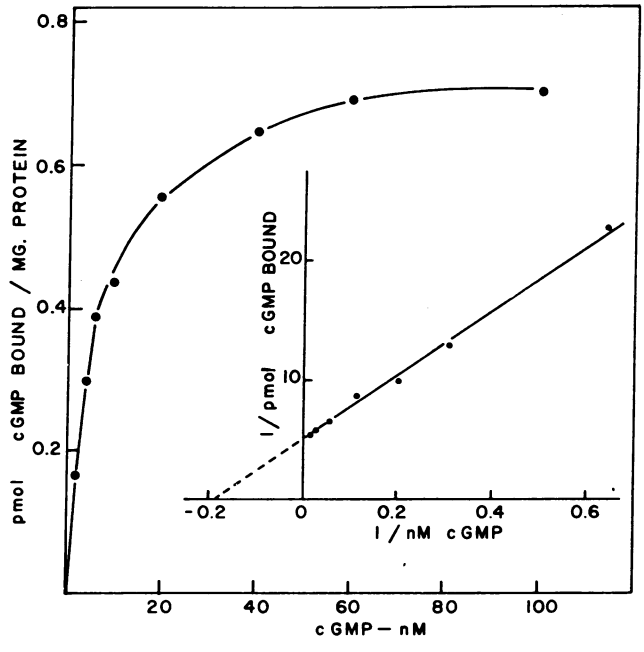

FIG. 1. Amount of cyclic GMP bound as a function of cyclic GMP concentration. Samples containing various concentrations of [^{3}H]cyclic GMP were incubated with 260 μg of enzyme protein at pH 4.0.

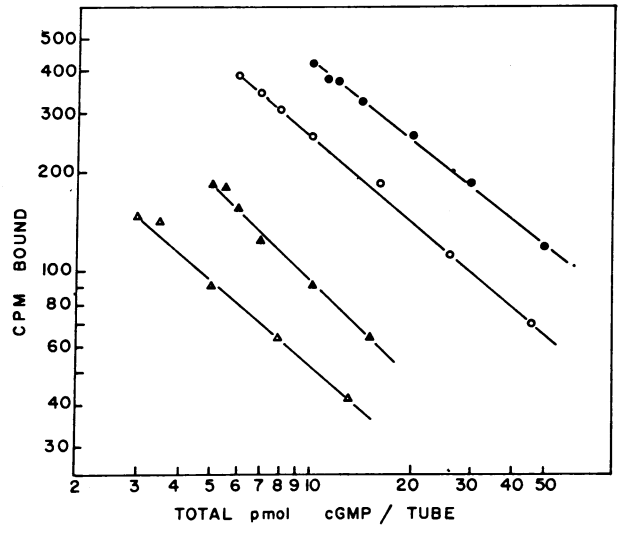

FIG. 3. Standard curves for cyclic GMP assay. Tubes containing either 10 (●) or 6 (○) pmol of [^{3}H]cyclic GMP in 0.1 ml, or 5 (▲) or 3 (△) pmol in 0.05 ml, were incubated as described in the text. Standard amounts of unlabeled cyclic GMP were added to some tubes as indicated. 260 μg of enzyme protein was used in the 0.1-ml incubations and 130 μg of protein with 0.05 ml. Values for cyclic GMP per tube represent total amounts (radioactive and unlabeled).

TABLE 1. *Effects of other nucleotides on the binding of cyclic GMP*

Nucleotide	Concentration	% Inhibition of [^{3}H]cyclic GMP binding
Cyclic GMP	0.2 μM	63
Cyclic GMP	0.05 μM	31
Cyclic AMP	1.0 μM	17
Cyclic AMP	0.1 μM	6
Cyclic IMP	10.0 μM	40
Cyclic IMP	1.0 μM	5
Cyclic XMP	10.0 μM	10
Cyclic XMP	1.0 μM	0
5′ AMP	1.0 mM	30
5′ AMP	0.1 mM	15

Samples containing 10 pmol (0.1 μM) of cyclic GMP and 260 μg of protein, in a total volume of 0.1 ml, were incubated as described in the text. Some tubes also contained either unlabeled cyclic GMP or another nucleotide as indicated. The values reported are the means of 2 or 4 incubations.

Presumably because of the nearly theoretical behavior of the system, the standard curves did not significantly differ from one day to another.

Effects of other agents on cyclic GMP binding

A protein has been described by Posner *et al.* (11) that inhibits cyclic AMP-stimulated protein kinase activity. This inhibitor enhances cyclic AMP binding and decreases the binding constant for cyclic AMP (7). A preparation of this protein inhibitor, which inhibited cyclic AMP stimulation of skeletal muscle protein kinase, did not alter cyclic GMP stimulation of lobster muscle protein kinase, nor did it alter cyclic GMP binding in this assay.

As summarized in Table 1, equimolar amounts of cyclic AMP inhibited cyclic GMP binding 5–7%. It was not until cyclic AMP was added in a 10-fold excess that interference became appreciable (15–20% inhibition). The binding constant for cyclic AMP was 0.1 μM, or about 15-fold higher than that for cyclic GMP. High concentrations (10 μM) of cyclic IMP and cyclic XMP also interfered with cyclic GMP binding. At this concentration, cyclic CMP, cyclic UMP, and cyclic TMP did not alter binding (not shown). High concentrations of 5′ AMP (1 and 0.1 mM) interfered with binding. ATP, ADP, adenosine, GTP, GDP, 5′ GMP, and guanosine, at 1 mM concentration, had little or no effect on binding when cyclic GMP was present at 0.1 μM (not shown). $CaCl_2$ (2.7 mM), KCl (6.5 mM), Mg (acetate)$_2$ (1.2 mM), and NaCl (14.5 mM) had no significant effect. However, 145 mM NaCl decreased binding by 35%.

Tissue concentrations of cyclic GMP and cyclic AMP

As summarized in Table 2, the values obtained for cyclic GMP and cyclic AMP in deproteinized extracts from cerebellum and lung were quite similar to values obtained after purification. With samples from liver, however, poor agreement was observed between assays performed before and after purification. Presumably, this is attributable to the relatively low concentrations of cyclic GMP in this tissue and the resulting inability to dilute out interfering materials in crude extracts prior to assay. For reasons that are not apparent, the cyclic AMP concentrations in these liver samples were considerably lower than those usually found (5, 16). Purification appeared to be unnecessary for assay of cyclic AMP and cyclic GMP in urine*. The infusion of calcium gluconate in a patient increased urinary excretion of cyclic GMP (Table 2) as reported previously (17).

DISCUSSION

The procedure described provides a simple, sensitive assay that requires considerably less time and effort than do several methods for cyclic GMP currently in use (2, 3, 5, 6). Furthermore, several of the previously described methods require routine purification of samples prior to assay. Although somewhat less sensitive than the immunoassay for cyclic GMP (5), an advantage of this binding assay over the immunoassay is the short time needed for preparation of a binding protein. Enough crude binding protein for 5000 or more determinations can be prepared in several hours.

Using [^{3}H]cyclic GMP of 4.45 Ci/mmol, and carrying out the incubation in a final reaction volume of 100 μl with 0.1 μM cyclic GMP, we can detect 1 pmol of cyclic GMP. If

TABLE 2. *Assay of cyclic GMP and cyclic AMP before and after purification of samples*

Tissue	Sample	pmol/mg protein: Cyclic GMP (*)	Cyclic GMP (†)	Cyclic AMP (*)	Cyclic AMP (†)
Lung	1	3.2	2.4	5.8	6.2
	2	3.7	3.1	6.2	7.7
Cerebellum	1	5.7	4.6	46.7	43.8
	2	2.9	2.6	34.6	28.4
Liver	1	2.4	0.23	2.0	2.5
	2	3.3	0.06	0.8	1.2
		μmol excreted/12 hr			
Human urine‡	1	0.29	0.35	3.5	3.4
	2	0.72	0.73	3.5	3.1
	3	0.33	0.35	...§	2.0

* Before purification on an ion-exchange column.
† After purification on an ion-exchange column.
‡ From a study done in collaboration with Dr. C. Y. Pak.
§ Not determined.

Urine samples and deproteinized tissue extracts were prepared as described in the text and assayed for cyclic GMP and cyclic AMP content before and after purification on Dowex AG 1 × 8 formate columns. Urine was collected in three consecutive 12-hr periods from a patient with hyperparathyroidism. At the beginning of the second collection period, the patient was given an intravenous infusion of calcium gluconate (15 mg Ca^{++}/gk) for 4 hr.

* It was also established by use of a liver phosphorylase activation assay (F. Murad, unpublished observations) that values obtained for cyclic AMP concentration in urine were similar before and after purification. Presumably, this is because many substances known to inhibit these assays (7, 16) are not found in urine. In addition, the relatively high concentrations of cyclic AMP in urine permit a 10- to 20-fold dilution before assay. There was excellent agreement of values for cyclic AMP when the same urine sample was analyzed by either liver phosphorylase activation or the binding assay.

radioactive cyclic GMP of greater specific activity were available, it would be possible to detect somewhat less than 0.5 pmol, but to increase the sensitivity of the method by much more, i.e., an order of magnitude, would require the availability of a binding protein with a greater affinity constant for cyclic GMP.

While it is possible to assay cyclic GMP in urine and deproteinized extracts of lung and cerebellum without purification, when concentrations are low purification will probably be necessary, as was the case with rat liver. As can be inferred from Tables 1 and 2, the actual tissue concentrations of cyclic GMP (and, hence, the relative ratio of interfering material to cyclic GMP), and not the ratio of cyclic AMP to cyclic GMP in the tissue, will determine whether purification is necessary. Although in cerebellar extracts, cyclic AMP concentrations were at least 10-fold greater than those of cyclic GMP, this ratio did not exist in the assay, which is itself carried out in the presence of a saturating concentration of cyclic GMP (0.1 μM). The chromatographic method described permits separation of 5′ AMP and cyclic AMP (materials that most interfered with cyclic GMP binding) from cyclic GMP. This method also results in essentially quantitative recovery of each cyclic nucleotide and, in most instances, determination of recoveries of each should not be necessary.

Whether the cyclic GMP-binding protein is in fact the cyclic GMP-stimulated protein kinase remains to be established. Attempts to answer this question have thus far been unsuccessful because of large losses of both activities with several attempted methods of further purification. For use in the cyclic GMP assay, however, further purification was not necessary.

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