A SIMPLE AND SENSITIVE COMPETITIVE PROTEIN-BINDING ASSAY FOR CYCLIC GMP

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Summary: A simple and sensitive competitive protein-binding assay for cyclic GMP (cGMP) has been developed, using a specific cGMP-binding protein isolated from rat lungs. The contamination of cAMP-binding protein was partially removed by fractionated precipitation with ammonium sulfate. The most active binding was observed when cGMP-binding protein was extracted with phosphate buffer (50 mM) containing 2-mercaptoethanol. Magnesium (40 mM) and zinc (4 and 40 mM) ions enhanced the binding of cGMP to the protein. The amount of protein which bound to cGMP decreased when the protein was extracted with buffer but without 2-mercaptoethanol. The binding constant of this protein was 5.5-16.3 x 10^{-9} M. The sensitivity of the assay was 0.2 picomole of cGMP. Other nucleotides exerted little or no interference.

The role of cAMP* as a second messenger of various hormone actions is well documented (1). Recently, acetylcholine (2), mitogenic agents (3), histamine, serotonin, bradykinin (4), and insulin (5) were reported to cause accumulation of cGMP in mammalian tissues without elevation of cAMP levels. A guanylate cyclase which catalyzes the formation of cGMP has been found in several mammalian tissues and has been shown to be different from adenylate cyclase (6). Furthermore, cGMP-dependent protein kinase activity has also been discovered in several mammalian tissues and arthropods (7, 8). It has been suggested that the cGMP system represents a new second messenger system independent from that of cAMP.

Although several methods are available to measure cGMP, they are laborious, expensive, or relatively insensitive. In the present paper, we describe a simple

* Abbreviations: Adenosine-3′,5′-monophosphate, cyclic AMP or cAMP; cytidine-3′,5′-monophosphate, cCMP; guanosine-3′,5′-monophosphate, cyclic GMP or cGMP; inosine-3′,5′-monophosphate, cIMP; thymidine-3′,5′-monophosphate, cTMP; uridine-3′,5′-monophosphate, cUMP; xanthosine-3′,5′-monophosphate, cXMP.

and sensitive protein-binding assay for cGMP. The procedure for the preparation of cGMP-binding protein from rat lung and the preliminary characterization of the protein are also included.

MATERIALS AND METHODS

Nucleotides and resin: ^{3}H-cGMP (21 Ci/mmole) was purchased from Amersham-Searle Corp., Arlington Heights, Ill., and ^{3}H-cAMP (37.7 Ci/mmole) was purchased from New England Nuclear, Boston, Mass. All unlabeled nucleotides were purchased from Sigma Chemical Co., St. Louis, Mo. Ion exchange resin, AG1-X2 (chloride form) was purchased from Bio-Rad Laboratories, Richmond, Calif.

Preparation of binding protein: The lungs were quickly removed from Sprague-Dawley rats (80-120 g) under ether anesthesia and rinsed in cold phosphate buffer (50 mM), pH 7.0, containing 2-mercaptoethanol (6 mM). Lungs from 10 to 20 animals in a batch were homogenized in 5 volumes of the same buffer with Ultra-Turrax (Janke & Kunkel KG), and the homogenate was filtered through 4 layers of gauze and centrifuged at 50,000x g for 60 min. Ammonium sulfate was added to a concentration of 22 g per 100 ml of the supernatant. The precipitate was discarded after centrifugation. An additional amount of ammonium sulfate (24 g per 100 ml of the original volume) was added to the supernatant. The precipitate collected after centrifugation was redissolved in the same buffer and contained cGMP-binding protein. The protein preparation was dialyzed against the same phosphate buffer (50 mM) containing 2-mercaptoethanol (6 mM) and glycerol (10%). The whole procedure was carried out at 0-4° C. The final preparation was analyzed for protein content by the method described by Lowry et al. (9) and stored in a freezer.

Protein-binding assay for cGMP: The cGMP-binding protein preparation was diluted in sodium acetate buffer (50 mM), pH 4.0. Any acid precipitable protein was removed by centrifugation. The binding assay for cGMP was carried out using the same acetate buffer (50 mM), pH 4.0, containing 1 picomole of ^{3}H-cGMP, 200-220 µg of binding protein, and either standard, other nucleotide or unknown sample in a final volume of 150 µl. When tested, divalent cations were dissolved in acetate buffer directly. After 60 min incubation at 0° C, 1 ml of Tris-HCl buffer (10 mM), pH 8.0, containing 40 mM $MgCl_2$ was added to stop the reaction (10). The contents of each assay tube were transferred to a cellulose filter (Millipore HAWP 02500), and the filter was washed with 5 ml of Tris-HCl buffer. The radioactivity retained by the filter was determined by liquid scintillation spectrometer (Packard Model 3390) in 10 ml of Bray's solution.

Kinetic study of cGMP-protein binding: Mixtures of 200 µg of cGMP-binding protein and varied amounts of ^{3}H-cGMP (1-50 picomoles) in a final volume of 200 µl of acetate buffer were incubated at 0° C for 60 min. The bound radioactivity was separated according to the same assay procedure employed for cGMP.

Determination of cAMP: Cyclic AMP was determined by the competitive protein-binding assay method described by Gilman (11), using a binding protein extracted from rat liver as described by Kumon et al. (12). Instead of Millipore filtration, a dextran-charcoal mixture was used to separate the free from the bound cAMP

Preparation of tissue samples: Cyclic GMP and cAMP contents in tissues were extracted and separated from each other by the method described by Mao and Guidotti, using alumina column prior to an ion exchange resin column (13).

RESULTS

The specificity of cGMP-binding protein isolated from rat lung is demon-

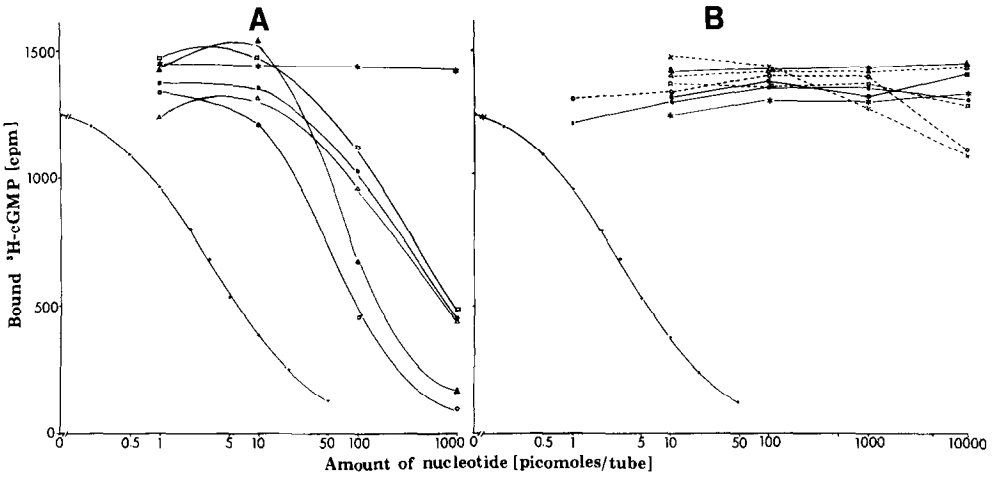

Fig. 1. A. Dose-binding curves of cyclic nucleotides, including cGMP (●——●), cAMP (○——○), cIMP (▲——▲), cXMP (△——△), cUMP (■——■), cCMP (□——□), and cTMP (*——*). B. Dose-binding curves of guanosine (▲——▲), 5′-GMP (■——■), GDP (*——*), GTP (◎——◎), adenosine (△---△), 5′-AMP (□---□), ADP (x---x), and ATP (○---○). As compared to that of cGMP (●——●), all nucleotides are inactive.

strated in Fig. 1-A and -B. A nearly complete inhibition of ^{3}H-cGMP binding to the protein could be achieved with 50 picomoles of unlabeled cGMP. The competition of binding could be detected with 0.2 picomole of cGMP. Of the various cyclic nucleotides examined (Fig. 1-A), cAMP and cIMP showed an appreciable cross-reaction, 1/15 and 1/26 that of cGMP, respectively. The other cyclic nucleotides had a much lower degree of competition, and cTMP did not compete at all. Guanosine, adenosine, and their phosphate derivatives other than the cyclic nucleotides, were totally inactive (Fig. 1-B).

Effects of divalent cations and 2-mercaptoethanol: When preparation of cGMP-binding protein was carried out in the presence of 2-mercaptoethanol (6 mM), the effect of some divalent cations on the binding of cGMP was tested, and the results are listed in Table 1. The binding of cGMP to protein was greatly enhanced by Zn^{2+} at both concentrations of 4 mM and 40 mM, and by Mg^{2+} only at 40 mM. The other divalent cations, namely Ca^{2+}, Mn^{2+} and Co^{2+}, exerted little influence. When buffer containing 50 mM of 2-mercaptoethanol was used to prepare the protein, the binding increased even without the cations. Under

TABLE 1

Effects of divalent cations and 2-mercaptoethanol on ^{3}H-cGMP binding to cGMP-binding protein

	Binding[1] (% of control)		
Cations[2]	4 mM	40 mM	
Mg^{2+}	105.7 ± 0.3	139.9 ± 0.5	
Ca^{2+}	104.5 ± 0.2	102.0 ± 0.2	
Mn^{2+}	103.9 ± 0.2	95.1 ± 0.2	
Co^{2+}	105.1 ± 0.2	95.1 ± 0.2	
Zn^{2+}	135.7 ± 1.3	158.6 ± 1.8	
2-Mercaptoethanol[3]	0	6 mM	50 mM
No cation	61.5 ± 0.5	100	144.1 ± 2.1
Mg^{2+}, 40 mM	41.5 ± 0.3	123.7 ± 0.8	112.8 ± 1.6
Zn^{2+}, 40 mM	45.9 ± 0.2	138.5 ± 1.0	177.8 ± 2.9

[1] The binding experiment was performed in triplicate, and the results are expressed as Mean ± 1 S.D.

[2] The experiment was carried out using cGMP-binding protein extracted with phosphate buffer containing 6 mM of 2-mercaptoethanol. The control system did not contain any cation, and 100% binding represented 0.112 picomole of ^{3}H-cGMP bound to 200 µg of protein.

[3] The cGMP-binding protein was extracted with phosphate buffer containing no 2-mercaptoethanol, up to the step of dialysis. Three aliquots of the preparation were dialyzed against buffer containing glycerol and specified concentrations of 2-mercaptoethanol. The control system used the protein dialyzed against buffer containing 6 mM of 2-mercaptoethanol and did not contain any cation. Control of 100% binding represented 0.12 picomole of ^{3}H-cGMP bound to 200 µg of protein.

these conditions, 40 mM of Mg^{2+} inhibited the binding, but 40 mM of Zn^{2+} maintained a stimulatory effect to further increase cGMP binding to protein. Protein extracted without 2-mercaptoethanol had much lower binding to cGMP, and neither Mg^{2+} nor Zn^{2+} exerted any enhancing effect.

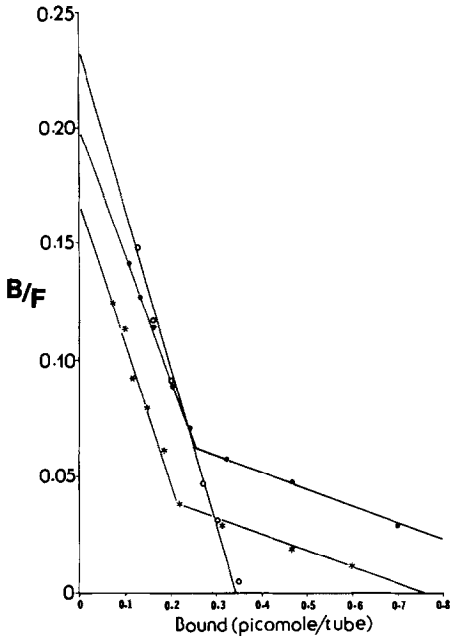

Fig. 2. Scatchard graphs of ^{3}H-cGMP binding. Control (*——*) has $KD_1 = 8.8 \times 10^{-9}$ M and $KD_2 = 0.9 \times 10^{-7}$ M. Mg^{2+} (●——●) does not affect the binding kinetics, as $KD_1 = 6.8 \times 10^{-9}$ M and $KD_2 = 0.9 \times 10^{-7}$ M. Zn^{2+} (o——o) affects the binding kinetics, exhibiting only a single KD = 7.4×10^{-9} M.

Kinetic studies: The kinetics of cGMP-protein binding were analyzed by Scatchard plotting and are illustrated by a typical example in Fig. 2. The protein contained two components for cGMP binding: one with high affinity (KD = 5.5-16.3 x 10^{-9} M), and the other with low affinity (KD = 0.9-2.0 x 10^{-7} M), as tested in 5 different batches of binding-protein preparations. These binding constants did not change in the presence of 40 mM of Mg^{2+}. When the binding experiment was performed in the presence of 40 mM of Zn^{2+}, the binding component of low affinity disappeared, while that of high affinity remained without any change in KD (7.4×10^{-9} M).

Tissue levels of cAMP and cGMP: The levels of cGMP in several rat tissues were measured by this competitive protein-binding assay. The same tissues were also used for cAMP determination. The results are listed in Table 2. The values of cGMP are similar to those reported previously by other investigators using different techniques (14-19). The values of cAMP are also in

TABLE 2

Assay of cGMP and cAMP levels in rat tissues[1]

Tissues	cGMP[2]	cAMP[2]	cAMP/cGMP
Lung	153.1 ± 10.3	420.0 ± 21.5	2.74
Liver	13.4 ± 1.5	176.5 ± 3.8	13.17
Heart	36.0 ± 3.1	395.0 ± 4.2	10.97
Testis	17.2 ± 1.0	328.5 ± 4.2	19.10
Epididymal fat pad	16.9 ± 3.0	67.5 ± 0.9	3.99
Kidney cortex	54.3 ± 3.8	611.9 ± 8.9	11.27

[1] Three different samples of each tissue were assayed in duplicate. The results are expressed as Mean ± 1 S.D.

[2] The values are picomoles/g wet weight of tissue.

the same range reported by others. In general, cAMP/cGMP ratios of various tissues are on the order of 10 to 20, with the exception of the lung and fat pad. Compared to other organs, the lung contains more cGMP.

DISCUSSION

Recent reports described cGMP-dependent protein kinase activity in many mammalian tissues, including rat and bovine cerebellum (20, 21), rat pancreas (22), guinea pig lung (23), and bovine adrenal cortex (24). From the bovine adrenal cortex, a cGMP-binding protein was partially separated from cAMP-binding protein using cAMP affinity column chromatography (25). The ever-increasing interest in a possible physiological role played by cGMP as second messenger warranted the development of a simple and sensitive method for the quantification of cGMP in tissue samples. Enzymatic methods for the assay of cGMP (14, 15, 19, 26) appeared to be time-consuming and tedious for routine measurement. The radioimmunoassay developed by Steiner et al. (27) depended

on the success of producing cGMP-specific antibody, which required immunization for a period of several months. Protein-binding assays using cGMP-binding protein from lobster muscle (16), or the activation of specific cGMP-dependent protein kinase (17), were less sensitive. In comparison, the competitive protein-binding assay developed by us offers many advantages over the other techniques. The extraction of cGMP-specific binding protein in large quantities can be accomplished in hours, and the assay procedure for a fair number of samples can be finished in a single day. The method is specific for cGMP, and specificity can be further increased if a simple pretreatment of tissue sample separating cAMP from cGMP is performed. The sensitivity of the method, which can measure as little as 0.2 picomole of cGMP, is considered adequate for general applications.

The cGMP-specific binding protein, isolated from rat lungs by simple fractionated precipitation with ammonium sulfate, is distinct from cAMP-binding protein. It has a binding constant of $5.5\text{-}16.3 \times 10^{-9}$ M, similar to that reported by Gill and Kanstein (25). The enhancement of cGMP binding by Mg^{2+} and Zn^{2+} is a unique feature of this particular cGMP-binding protein, since cAMP-binding protein prepared from rat liver by the method of Kumon et al. does not exhibit the same effect of divalent cations (12).

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