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CYCLIC GMP BINDING PROTEIN OF RAT HEART AND URINARY CYCLIC NUCLEOTIDES IN PATIENTS WITH DIABETES MELLITUS BEFORE AND AFTER TREATMENT

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INDEXING WORDS

cGMP binding protein; cAMP; cGMP; diabetes mellitus

SYNOPSIS

Binding capacities of rat heart muscle protein to cyclic AMP and cyclic GMP were examined.

A competitive protein binding assay for cyclic GMP was developed, using 55% ammonium sulfate fraction of the homogenate of the heart muscle, in combination with Dowex 1x8 column chromatography.

Urinary cyclic AMP and cyclic GMP were measured in 7 patients with diabetes mellitus before and after treatment. Cyclic GMP/creatinine decreased in 5 patients after treatment and cyclic AMP/creatinine decreased in the patients with good control.

INTRODUCTION

The accumulation of guanosine 3',5'-monophosphate (cGMP) in response to some stimulations has been reported to occur in a number of biological systems, different from the change of cyclic adenosine 3',5'-monophosphate (cAMP).[11)]

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Cyclic GMP dependent protein kinase (G-kinase) has been recently described in several mammalian tissues,[13,17,20,31] and cGMP binding activity has been separated from cAMP receptor protein.[5,7,21,27]

In this paper, some characters of 0-55% ammonium sulfate fraction of rat heart homogenate were examined and a competitive binding assay for cGMP was developed using this fraction.

Changes of urinary cyclic nucleotides in patients with diabetes mellitus before and after treatment were also reported.

MATERIALS AND METHODS

Tissue Preparation.

Male Wistar rats were decapitated and tissues were quickly removed and rinsed in cold 4 mM EDTA (pH 7.0). The tissues were homogenized in 6 volumes of the same solution in a small Waring blendor,and centrifuged at 27,000 x g for 60 minutes at 4°C.

Ammonium Sulfate Fractionation.

Neutral saturated cold ammonium sulfate solution was added to the supernatant of the heart muscle to attain 55% saturation. The precipitate was collected by centrifugation and resuspended in a small amount of 5 mM potassium phosphate buffer (pH 7.0), then dialysed against the same buffer.

DEAE-Sephadex A-50 Column Chromatography.

A column (1 x 20 cm) was equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) as previously described.[25]

Purification of Urinary Cyclic Nucleotides.

Urine was diluted 2-5 folds with 0.05 M Tris-formate buffer (pH 7.5) containing a tracer dose of ^{3}H-cGMP and loaded to a 0.5 x 2 cm neutral alumina (Merck, activity I) column put above a 0.5 x 3 cm Dowex 1x8-formate column. Cyclic nucleotides were eluted from the alumina column with 5 ml of the same buffer, being successively loaded on the Dowex column. After drained, the Dowex 1x8 column was washed with 10 ml of water, followed by the elution of cAMP with 10 ml of 1 N formic acid, and the elution of cGMP with 10 ml of 4 N formic acid. Each fraction was lyophilized and dissolved in 50 mM acetate buffer. A portion was mixed with Bray's solution and radioactivity was measured by a liquid scintillation counter for calculation of recovery rate.

Urinary Creatinine.

Urinary creatinine was measured by the Jaffe's reaction.[2)]

Assay Procedures.

The assay system for binding reaction contained 50 mM acetate buffer (usually pH 4.0), various amount of ^{3}H-cGMP or ^{3}H-cAMP, cyclic nucleotide (10^{-8}-10^{-7} M) and binding protein in 80 µl. The mixture was incubated at 4°C for 120 minutes.

In the method A, 1 ml of 20 mM potassium phosphate buffer (pH 6.2) was added and the mixtures were passed through Millipore membrane filters. The filters were washed with 10 ml of the phosphate buffer and dissolved in 10 ml of Bray's solution.

In the method B, 1 ml of 60% ammonium sulfate was added to the mixture and passed through the filters, then washed with 10 ml of the same ammonium sulfate solution. The filters were rinsed with 0.5 ml of 10% sodium dodesyl sulfate and put at 25°C for 15 minutes. Then 2 ml of Methyl Cellosolve, 2 ml of BIO-SOLVE (BBS-3, Beckman Co.) and 10 ml of Toluene Scintillator Solution were added and the filters were dissolved. Radioactivity was measured in a liquid scintillation counter.

RESULTS

Binding capacities of several tissues to cAMP and cGMP have been examined, using the 27,000 x g supernatant (Fig. 1). Skeletal muscle showed the highest binding to cAMP. The highest binding to cGMP was observed in the supernatant of the heart muscle. Therefore, this supernatant was further subjected to the ammonium sulfate fractionation (55%). The dialysed fraction was used in the following study, if not otherwise indicated.

In the step of the filtration with Method A, some variations have occurred, depending upon the time elapsed after the addition of the phosphate buffer before the filtration (Table 1). Therefore Method B was used because of minimum variation (data not shown).

The binding reaction to cGMP reached to maximum at 150 minutes at pH 4.0, 4°C and was followed by a slight reduction of the binding (Fig. 2).

The amount of bound cGMP was proportional to the amount of

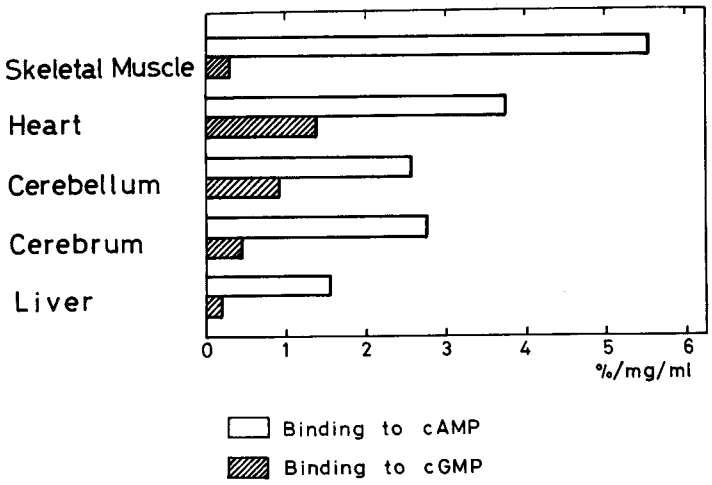

Fig. 1 Binding capacities of the 27,000 x g supernatant of rat tissues to cAMP and cGMP.
Binding capacity was conveniently expressed as % bound counts of the total radioactivity added (1 - 6 pmoles of the ^{3}H-cyclic nucleotides)/mg protein/ml of the supernatants. Incubation was performed at pH 4.0 and reaction was terminated by the method A with immediate filtration (see Text).

Table 1 Variation by filtration with potassium phosphate buffer.

Additions	0		cGMP	2.5 x 10^{-7}M
Filtered	3476		1877	
immediately	3276	3389 ± 48*	1776	1807 ± 28**
	3415		1769	
Filtered	2995		1679	
5 min. later	2359	2649 ± 152*	1267	1482 ± 97**
	2592		1501	

Values are cpm. *mean ± S.E. and the difference is significant ($p<0.02$). **$p>0.1$.

protein added (Fig. 3). The effect of pH on the binding of cGMP to the protein is shown in Fig. 4. The highest affinity was seen at pH 4.0.

The apparent specificity of the nucleotide binding to the protein was examined by the competition of several cyclic nucle-

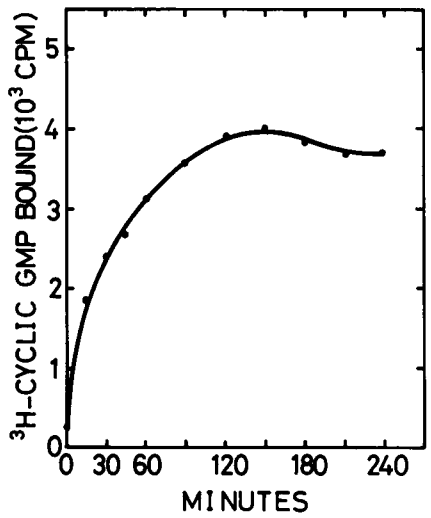

Fig. 2 Time course of binding reaction.
Each tube contained 4 μmoles acetate buffer (pH 4.0), 6 pmoles ^{3}H-cGMP (12,000 cpm) and 285 μg binding protein in 80 μl. Incubation was performed at 4°C. By the method B (see Text), the reaction was terminated at the time indicated and radioactivity was measured.

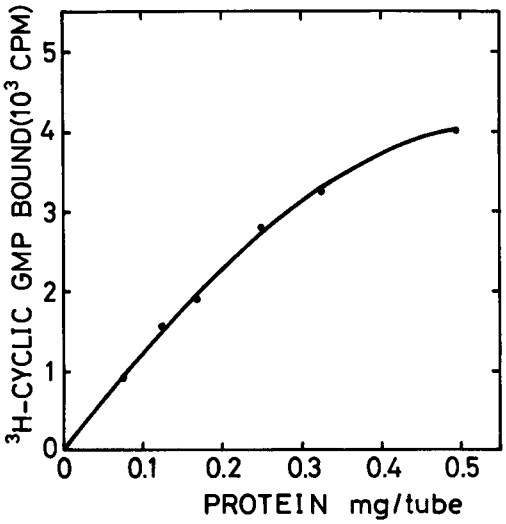

Fig. 3 Binding of cGMP to 55% ammonium sulfate fraction.
Varying amounts of the dialysed 55% ammonium sulfate fraction (see Text) were added. Other conditions were the same as stated in the legend of Fig. 2.

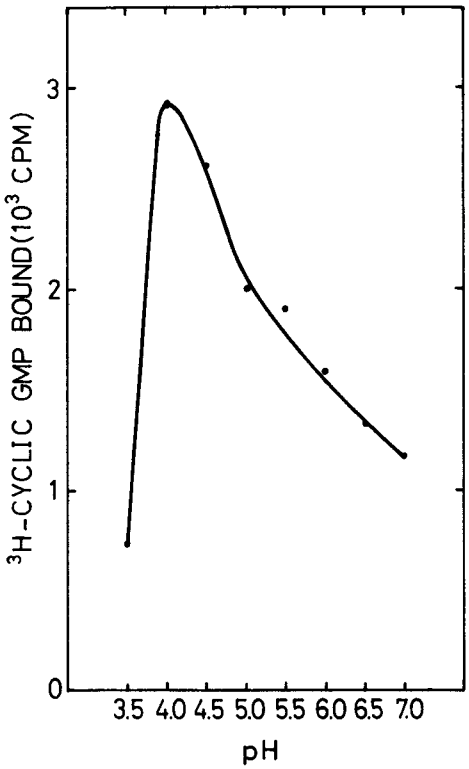

Fig. 4 Effect of pH on cGMP binding.
PH was adjusted with 50 mM sodium acetate buffer at final concentration. Other conditions were the same as stated in the legend of Fig. 2.

otides (Fig. 5). This crude protein fraction showed the most affinity to cAMP. Inosine 3', 5'-monophosphate which structurally resembled cAMP was an effective competitor for cGMP binding. Cyclic 3', 5'-uridine monophosphate also showed a competition to less extent.

Double reciprocal plots of the binding reactions for cAMP and cGMP are shown in Fig. 6. The bindings of these nucleotides appeared to be competitive each other at the higher concentration of the nucleotides. There were two apparent affinity constants for cGMP, namely high affinity Ka=2 x 10^{-8} M and low affinity Ka=2 x 10^{-6} M.

From these data, further purification seemed necessary for the study of the binding kinetics.

An attempt was made to purify the cGMP binding protein using

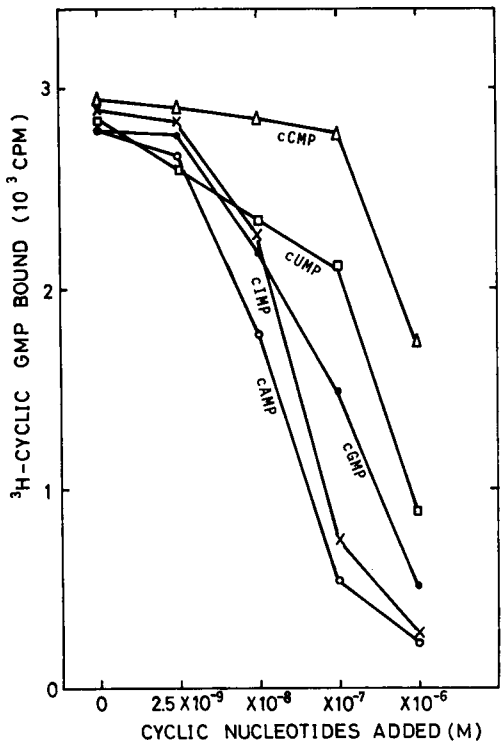

Fig. 5 Competitive binding of various cyclic nucleotides to 55% ammonium sulfate fraction.
Each tube contained 4 μmoles of acetate buffer (pH 4.0), 2 pmoles of ^{3}H-cGMP and various kinds of cyclic nucleotides to give the final concentration indicated. After incubation for 120 minutes at 4°C, the filtration was done by the method B (see Text).

the DEAE-Sephadex A-50 column, which has offered a sequential separation of a protein, depending upon pH as shown with the serum protein.[25] The ammonium sulfate fraction was successfully purified and separated into two peaks, but the binding for cGMP was almost lost though a little activity remained in the second peak of cAMP binding protein (Fig. 7).

An application of the ammonium sulfate fraction to the assay for cGMP was made with the combination of the alumina column and the Dowex 1x8 column to estimate urinary cyclic nucleotides in patients with diabetes mellitus before and after treatment.

Recovery rate throughout the column was 46.6 ± 7.5 (mean ± S.E.)%.

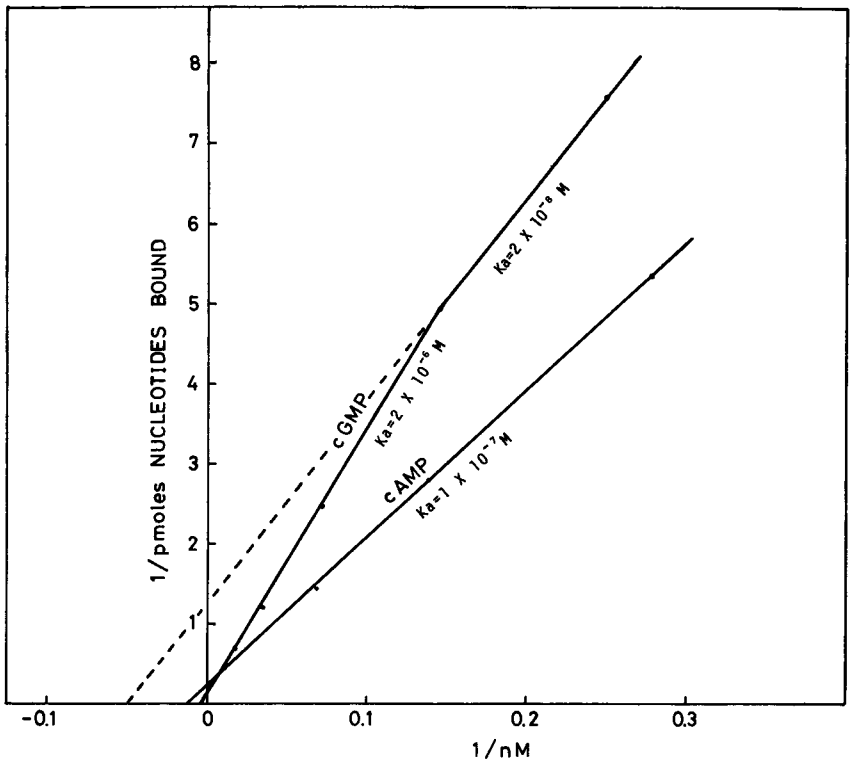

Fig. 6 Double reciprocal plots for cAMP and cGMP binding. Varying amounts of ^{3}H-cAMP and ^{3}H-cGMP were incubated with 285 μg of 55% ammonium sulfate fraction at pH 4.0, 4°C for 120 minutes. The method B was used for the filtration (see Text).

Recovery of endogenous and added cGMP from urine was satisfactory as shown in Table 2.

A standard curve for cGMP assay is shown in Fig. 8.

Cyclic AMP was measured with the modified method[26] of Gilman's competitive protein binding assay[9] without column chromatography.

Preliminary data on diabetic patients are shown in Table 3. Urinary cAMP, expressed as μmoles/g creatinine, decreased after the treatments in 5 out of 7 patients examined. Urinary cGMP (μmoles/g creatinine) decreased in 5 out of 7 patients and seemed to have paralleled the changes of cAMP in the patients with good

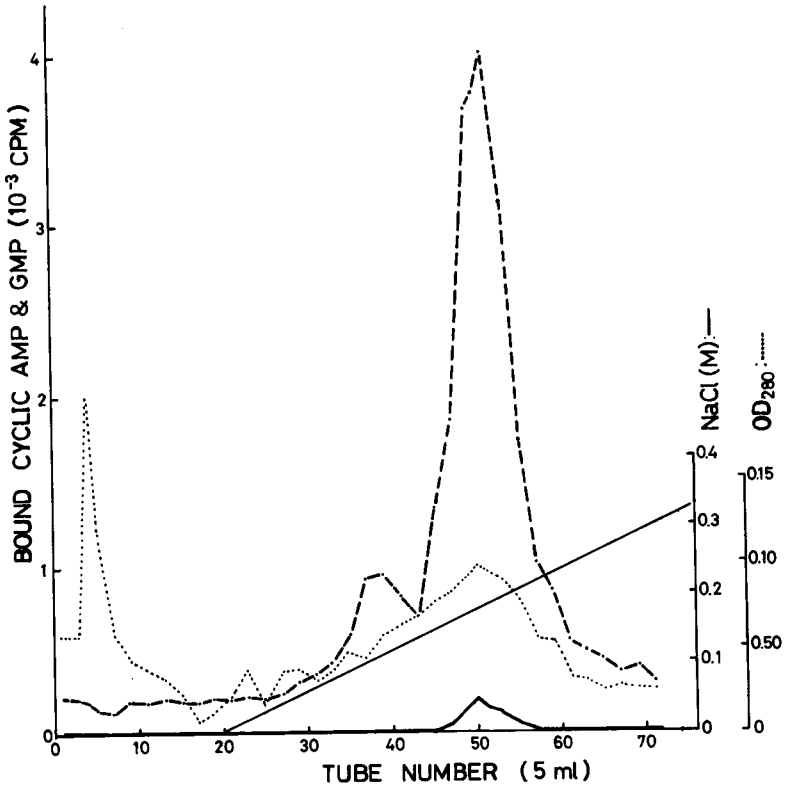

Fig. 7 Further purification of 55% ammonium sulfate fraction. The dialysed 55% ammonium sulfate fraction was applied to the DEAE-Sephadex A-50 column (see Text). Binding capacities were analysed by the system containing 1 pmole of ^{3}H-cAMP (12,000 cpm) or 6 pmoles of ^{3}H-cGMP (12,000 cpm), 15 µmoles of acetate buffer (pH 4.0) and 100 µl of each fraction in 170 µl of total volume, incubated for 120 minutes at 4°C. The reaction was terminated by the method A with immediate filtration.

Table 2 Recovery of endogenous and added cyclic GMP from urine.

Urine Volume (ml)	cGMP added	Measured value	Expected value	% of expected value
0.2	0	62		
0.4	0	136		
0.2	100	165	162	102%
0.2	200	252	262	96%

The values are pmoles.

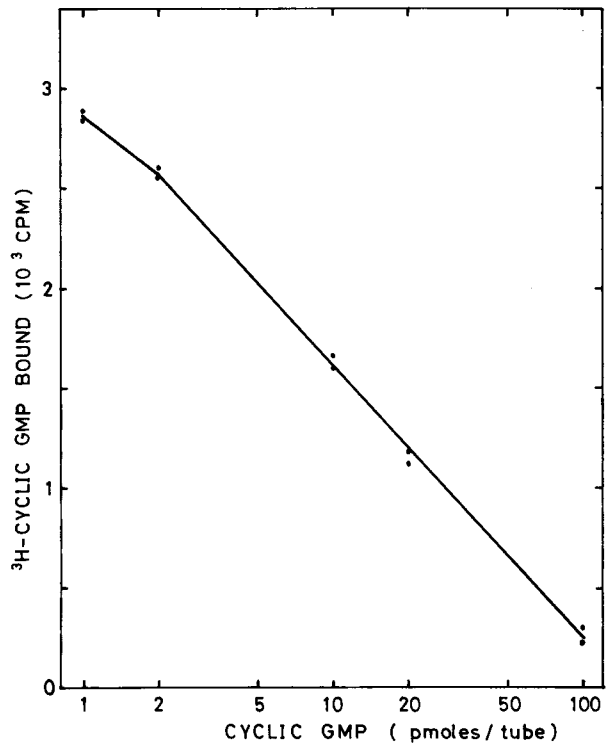

Fig. 8 A standard curve for the assay of cGMP. Each tube contained 2 pmoles of ^{3}H-cGMP and incubated for 120 minutes. Other conditions were the same as stated in the legend of Fig. 2.

control. Reciprocal changes were seen in the patients with fair control.

DISCUSSION

As shown here, the heart muscle revealed the highest binding activity to cGMP among the several tissues which we examined in the rat. Lincoln et al. reported that the lung showed the highest activity in the rat.[19] Physiologically cGMP may play role(s) in the heart and such organs containing relatively high concentration of the nucleotide and high cGMP binding activity as lung, cerebellum and intestine.[18,19,24]

Compared with the cAMP binding protein reported by Gilman,[9] relatively loose binding of the protein to cGMP is apparent from

Table 3 Changes of urinary cAMP and cGMP in patients with diabetes mellitus before and after treatment.

Case No	age (yr)	sex*	date	FBS** (mg/dl)	Urine Volume (ml)	cAMP/crn*** (μmoles/g)	cAMP/24-hr (μmoles)	cGMP/crn (μmoles/g)	cGMP/24-hr (μmoles)	Treatment (daily)	Assessment of Treatment
1	55	m	Jul.21	208	1000	4.63	0.230	0.774	0.384	diet (1800 Cal)	
			Aug.10	98	1400	0.03	0.014	0.153	0.081	+ Lente Insulin (20 u)	good
2	34	m	Aug.31	211	1750	1.35	1.268	0.512	0.482	diet (1800 Cal)	
			Sept. 1	206	1350	1.23	1.283	0.416	0.433		
			Sept. 3	192	2600	0.82	1.609	0.795	0.399		
			Sept. 4	205	2200	0.64	0.990	0.465	0.722		
			Sept.20	101	700	0.76	0.875	0.526	0.602	+ Tolbutamide (1.5 g)	good
3	25	m	Sept. 4	167	1000	1.19	0.590	0.929	0.462	diet (1800 Cal)	
			Sept.20	93	900	0.70	0.760	0.607	0.558	+ Tolbutamide (1.5 g)	good
4	57	m	Jul.28	178	1600	1.60	1.680	0.397	0.416	diet (1800 Cal)	
			Jul.29	180	1600	2.16	2,880	0.492	0.657		
			Aug.28	136	1400	1.49	0.980	0.542	0.309	+ Tolbutamide	fair
			Aug.29	128	1300	9.71	2.438	1.764	0.443	(0.5 g)	
5	47	f	Jul.25	218	1000	2.58	1.600	0.968	0.601	diet (1600 Cal)	
			Jul.26	237	1400	1.19	0.945	0.797	0.631		
			Aug.28	129	1000	1.41	0.700	0.666	0.397	+ Acetohexamide	good
			Aug.29	116	200?	1.39	0.150?	0.730	0.079?	(0.5 g)	
6	55	f	Aug. 2	204	1000	2.23	1.400	0.731	0.505	diet (1600 Cal)	
			Aug.30	134	800	2.00	1.400	0.520	0.373	+ Acetohexamide	fair
			Aug.31	146	600	2.24	1.360	0.566	0.258	(0.5 g)	
7	47	f	Jul.21	157	900	0.94	0.540	1.558	0.890	diet (1600 Cal)	
			Aug.28	137	1300	1.56	0.487	0.840	0.263	+ Acetohexamide	fair
			Aug.29	128	700	1.33	0.385	0.641	0.186	(1.0 g)	

*m: male, f: female
**FBS: fasting blood sugar
***crn: creatinine

the data in Table 1. The double reciprocal plots showed a deviation from lineality, suggesting the existence of two binding sites with different affinities. At the higher concentration of cGMP, a competitive type of inhibition by cAMP was observed. This is compatible with our data that the binding activity to cGMP has been purified on DEAE-Sephadex A-50, corresponding to the peak of cAMP binding. Concentrations of cGMP in tissues are one tenth to twentieth of cAMP.[18,24] Therefore, the binding protein with high affinity constant seems to be of physiological significance.

Murad et al. attempted to purify cGMP binding activity from lobster tail muscle without success.[22] Hofmann and Sold also tried to purify G-kinase from cerebellum on DEAE-Cellulose and other columns but the activity was almost lost by the purification.[13] In the course of this experiment, however, Takai et al. of our university reported to have succeeded in the partial purification of G-kinase and cGMP binding activity from bovine cerebellum, employing dithiothreitol in the buffer on a DEAE-Cellulose column.[27] Gill and Kanstein also purified cGMP receptor protein by affinity chromatography from bovine adrenal gland. Its dissociation constant was 1.4×10^{-8} M.[8] They also stated that the lower affinity constant (Kd=7×10^{-7} M) of the 0-50% ammonium sulfate fraction of bovine adrenal cytosol presumably represented cGMP binding to the cAMP receptor site. This may agree with our data shown in Fig. 6.

PH in the binding assay of cyclic nucleotides is an important factor. Haddox et al. have reported that cGMP binds to some forms of cAMP-dependent protein kinases with high affinity at acidic pH.[8] Our data coincided with their results.

Recently Gill et al. succeeded in the G-kinase purification from bovine lung to homogeneity.[8] It has an apparent molecular weight of 150,000 composed of 2 subunits of 74,000 molecular weight and Kd=2.2×10^{-8} M, which is similar to the high affinity constant of our experiment.

de Jonge and Rosen have recently documented that G-kinase is a substrate for its own phosphotransferase activity and that other protein substrate and cAMP modulate the process of self-phosphorylation.[16] Cyclic GMP has also been reported to increase the rate of cAMP hydrolysis by phosphodiesterase.[1] These evidences strongly suggest that cAMP and cGMP have close interaction in regulation of cellular metabolism.

There are two kinds of reported cyclic nucleotides in human urine, namely cAMP and cGMP. Radioimmunoassay may be more excellent than competitive binding assay with binding protein. In this report, however, the ammonium sulfate fraction was adopted for the assay of urinary cyclic nucleotides with the combination of the column chromatography. The columns yielded satisfactory separation of the cyclic nucleotides.

Urinary cyclic nucleotides change in various conditions.[23] Tucci et al. reported that urinary cAMP/creatinine ratios were significantly higher in the diabetic patients before treatment and returned to normal after treatment.[29] Diabetes mellitus is considered to be a bihormonal disorder involving a relative or absolute deficiency of insulin and inappropriate hyperglucagonemia.[30]

In the case No. 1, marked decrease in both nucleotides was observed after the treatment with insulin. This is consistent with the report that the administration of insulin was accompanied by decreased intracellular cAMP in liver and adipose tissue.[4,6] Concentration of cGMP in these tissues, however, was reported not to be influenced by insulin,[4,6] except one report that insulin increased cGMP in isolated fat cells.[14] Thompson et al. have shown that membrane bound high affinity cAMP phosphodiesterase of diabetic rats was stimulated by insulin administration and cGMP phosphodiesterase was not affected.[28]

There occurred the decreases in cGMP excretion in 5 cases out of 7 patients after various kinds of treatments for the diabetes in our study. Other factors might have changed after the treatment.

Tolbutamide is a known phosphodiesterase inhibitor.[3] With the treatment of this drug, excretion of the nucleotides was expected to rise, but no increase was observed in the majority of the cases.

Comparison of several drugs in the treatment of diabetes mellitus in more patients may offer information on the metabolism of the nucleotides in this pathophysiological condition.

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