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# OCCURRENCE OF THE METHYLISOBUTYLXANTHINE-STIMULATED CYCLIC GMP BINDING PROTEIN IN VARIOUS RAT TISSUES

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SUMMARY: A new type of cGMP binding protein, the activity of which is characteristically stimulated by methylisobutylxanthine, has been previously discovered in rat lung and platelets (Hamet, P. and Coquil, J.F. (1978) J. Cyclic Nucleotide Res. 4, 281-290). In the present study, we demonstrate the occurrence of this protein in soluble extracts of a variety of rat tissues fractionated by a DEAE-Sepharose chromatography. In several tissues (spleen, lung and brain) the binding activity of this protein was of the same order of magnitude as that of the cGMP-dependent protein kinase. 

As an intracellular regulator, cGMP is believed to be involved in the control of a variety of biological processes (1). There is a lot of evidence suggesting that the cGMP-dependent protein kinase has an important role in the expression of the physiological effects of cGMP (2). However, several studies have shown that certain tissues, namely, rat and human platelets (3-5), rat and bovine lung (3,6,7), smooth muscle cells of rat aorta (4) and sea urchin sperm (6) contain a high specific cGMP binding activity which is not related to the protein kinase but is associated with a cGMP phosphodiesterase. Furthermore, a similar cyclic GMP binding, due to a cyclic GMP phosphodiesterase has been found in rod outer segments of frog retina (8). Various observations strongly suggested that this binding did not occur at the catalytic site of the phosphodiesterase. Biochemical and immunological evidence showed, however, that these cGMP binding protein-phosphodiesterases(cGBPP) are distinct from the cGMP-stimulated phoshodiesterase (7,9) which is believed to possess a regulatory site specific for cGMP (10,11). At present, the function of the non catalytic site of cGBPP remains unknown. Nevertheless, certain data suggested that it might mediate a part of the biological actions of cGMP. Thus, an important binding of cGMP happened on cGBPP at physiological concentrations of the nucleotide (3-8), and in some tissues (platelets, rod outer segments of frog retina and sea urchin sperm) cGBPP appeared to be the major "receptor" for cGMP (3-6,8).

The binding activity of cGBPP has been shown to be stimulated by methylisobutylxanthine, an inhibitor of phosphodiesterase, and greatly decreased

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by incubation at acidic pH. Using these properties, in the present studies, we demonstrate the occurrence of cGBPP in extracts of various rat tissues after fractionation on DEAE-Sepharose column.

## MATERIALS AND METHODS

Nucleotides and nucleosides were purchased from Boehringer Mannheim. [$^3$H]cGMP (15.1 Ci/mmol) was obtained from Amersham (France) and 1-methyl-3-isobutylxanthine from Aldrich Chemicals. Bicine (N, N-bis 2-hydroxyethyl glycine) was provided by Calbiochem and DEAE-Sepharose CL-6B by Pharmacia Fine Chemicals. Cellulose ester filters (0.45 µm) were supplied by Gelman Instrument or by Millipore.

Tissues from male Wistar rats (200-300 g) were carefully rinsed and homogenized in 4 volumes (4 ml/g of tissue) of a buffer solution (buffer A) consisting in 20 mM sodium phosphate, pH 7.0, 2 mM EDTA and 25 mM 2-mercaptoethanol. The homogenate was centrifuged for 30 min at 30 000 x g and the supernatant obtained was adjusted to 66% $(NH_4)_2 SO_4$. The precipitate was collected by centrifugation (30 000 x g, 30 min), taken up in buffer A and dialyzed overnight against the same buffer. The enzyme preparation was applied to a DEAE-Sepharose column (1.6 x 20 cm) pre-equilibrated with buffer A. The column was washed with 6 volumes of buffer A and eluted with a linear gradient of NaCl (0 to 0.6 M ; total volume, 600 ml). Fractions of 8 ml were collected. The same procedure or similar ones have been previously used to partially purify the cGBPP from lung or platelets (3-5,9). The cGMP binding activity was measured as described (4). The standard assay mixture (total volume, 0.1 ml) contained 50 mM Bicine-NaOH buffer, pH 9.0, 0.1 µM [$^3$H] cGMP and 0.5 mg.ml$^{-1}$ bovine serum albumin. Reactions were initiated by the addition of 65 µl of column fractions containing 2 mM EDTA and 25 mM 2-mercaptoethanol. Tubes were incubated at 0°C for 120 min. Then, assay mixtures were passed through cellulose ester filters (0.45 µm). The filters were washed with 50 ml of cold buffer and dissolved in 1 ml of cellosolve prior to counting in a liquid scintillitation spectrometer. The blank values which were determined by incubating assay mixtures without biological samples were lower than 50 cpm. Phosphodiesterase and cGMP-dependent protein kinase were assayed following the procedures previously described (3,4).

## RESULTS AND DISCUSSION

As previously shown for lung, platelets and aortic smooth muscle cells (3-6), DEAE-Sepharose chromatography of the soluble fraction of seven other rat tissues resolved one major peak of cGMP binding activity stimulatable by methyl-isobutylxanthine (peak I) (Fig.1). In all tissues this peak was eluted at the same NaCl. concentration about (0.14 M) and just before another peak not sensitive to methyl-isobutylxanthine (peak II). Measurement of cGMP-dependent protein kinase activity indicated that peak II was due to this enzyme as it could be expected from preceding studies (3,4,6,7). The relative height of peak II in the different tissues essentially corresponded to the tissular distribution previously reported for the cGMP-dependent protein kinase (15,16). To further identify peak I, cGMP binding activity in the column fractions was measured at pH 6.0 instead of pH 9.0 and the elution profile of cGMP phosphodiesterase activity was determined. In conformity with the known characteristics of cGBPP (3-6), in every tissue the binding activity of peak I was nearly abolished by incubation at pH 6.0 and a peak of cGMP phosphodiesterase activity was found in the same column fractions (data

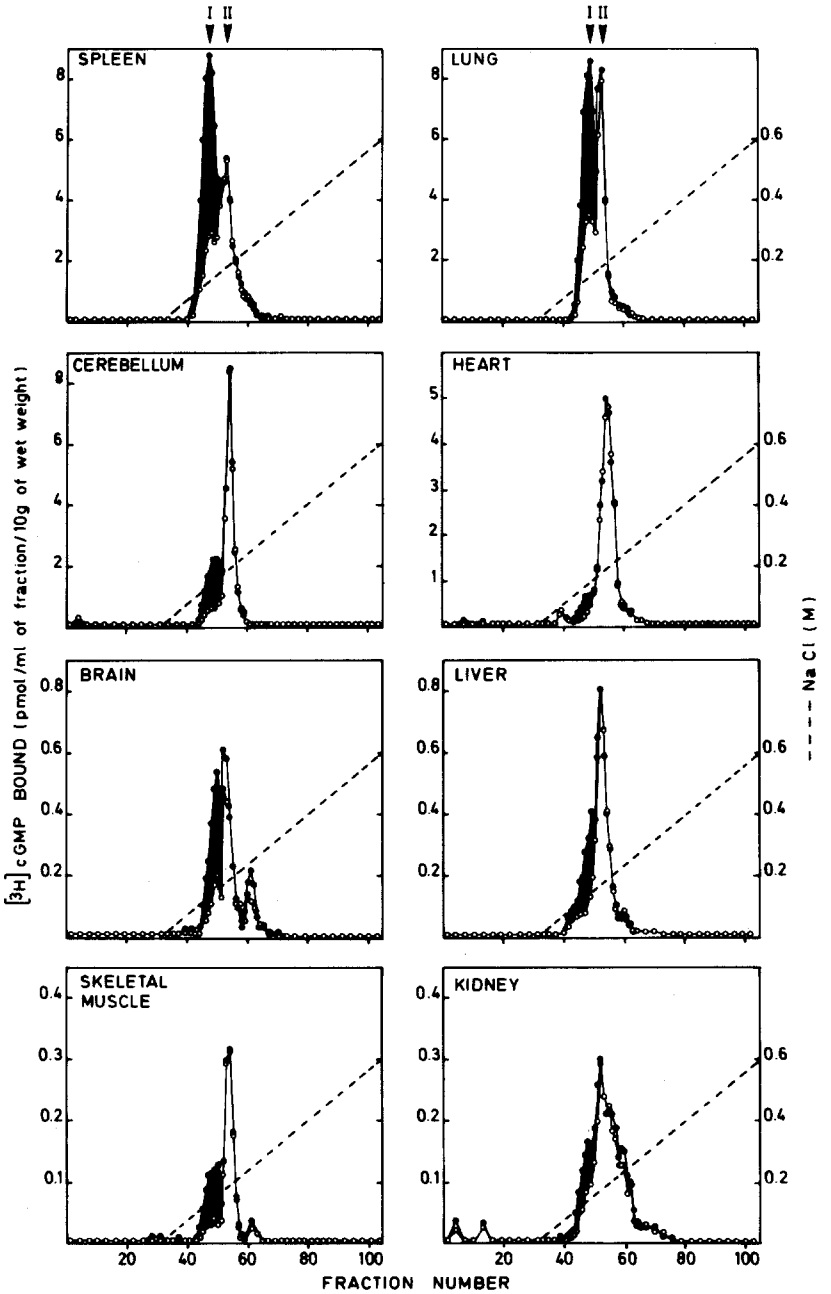


Fig. 1: Elution profiles of [$^3$H]cGMP binding activity on DEAE-Sepharose columns of extracts from various rat tissues. Ten to 20 g of tissue except for cerebellum (3.3 g) and liver (54 g) were homogenized in 4 volumes of buffer A. The 30 000 x g supernatants were concentrated by precipitation with 66% $(NH_4^+)_2$ $SO_4$ and the enzyme preparations were applied to DEAE-Sepharose columns (1.6 x 20 cm) pre-equilibrated with buffer A. Columns were washed with 6 bed volumes of buffer A and eluted with a linear gradient of NaCl from 0 to 0.6 M in the same buffer. Fractions of 8 ml were collected. [$^3$H] cGMP binding activity was measured in 50 mM Bicine-NaOH buffer (pH 9.0) at 0°C with (●) or without ( ○ ) 2 mM methyl-isobutylxanthine. The increase of cGMP binding activity induced by this agent in peak I is shown as a black area.

not shown). The small peak of cGMP binding activity or the tail at the end of peak II, depending on the tissue, (fractions 59 to 62, Fig. 1) appeared to be due to the cGMP-stimulated phosphodiesterase which was found in the same fractions. Martins et al. (11) have demonstrated the binding of cGMP by this type of phosphodiesterase purified from bovine adrenal and heart tissues. Finally, determination of cGMP binding activity in the column fractions at pH 6.0 or 7.5 did not reveal new peak in any tissue studied in addition to those seen at pH 9.0.

While cGBPP was found in every rat tissue, its level was variable. The highest concentrations of cGBPP were measured in spleen, lung and cerebellum and the lowest in skeletal muscle and kidney (Table I). Earlier studies on the cGBPP have shown that the cGMP binding activity in crude extracts of rat platelets which contain primarily cGBPP is much higher than that in rat lung (4,14). Thus, platelets appear to be the richest source of cGBPP among the rat tissues studied up to now. The tissular distribution of the cGBPP is very much alike the one reported for cGMP. Thus, high levels of this cyclic nucleotide have been measured in lung, cerebellum, spleen (15) and platelets (16) as compared with liver, kidney and skeletal muscle (15). In accordance with this relationship observed in rat, both cGMP and cGBPP have been found to be highly concentrated in rod outer sebments of frog retina (8,17) and sea urchin sperm (6,15).

TABLE I : Maximal increase of cGMP binding activity induced by methylisobutylxanthine in peak I from the different rat tissues

| TISSUE | INCREASE OF cGMP BINDING ACTIVITY pmol [$^3$H]cGMP bound/ml/10 g tissue |
|---|---|
| Spleen | 6.00 |
| Lung | 5.10 |
| Cerebellum | 1.59 |
| Heart | 0.44 |
| Brain | 0.33 |
| Liver | 0.20 |
| Skeletal muscle | 0.10 |
| Kidney | 0.07 |

Data were derived from profiles in Fig. 1. Depending on the profiles the maximal increase of cGMP binding activity was measured in fractions 47 to 50.

The relative binding activity of cGBPP to cGMP-dependent protein kinase also varied from tissue to tissue. While human and rat platelets (3-5) as sea urchin sperm (6) have been found to contain primarily cGBPP, this study showed that in spleen, lung and brain from rat,the activities of the two binding proteins are of the same order of magnitude. This has been previously reported for rat lung (3,6). Finally, a much higher level of binding activity due to the protein kinase was measured in extracts from heart and kidney..Evidence accumulated to date indicates that cGBPP is a cGMP phosphodiesterase distinct from the two well-recognized major forms of this enzyme i.e. the calmodulin-sensitive and the cGMP-stimulated phosphodiesterases (5-7,9). The present results suggest that, as these two forms, cGBPP might be widely distributed. The lack of earlier demonstration of this cGMP phosphodiesterase in a variety of tissues is likely due, in a great part, to the fact that its elution profile from DEAE-cellulose column is very close to that of the calmodulin-dependent phosphodiesterase (7).

The data presently available do not support the idea that all effects of cGMP are mediated by cGMP-dependent protein kinase (18). Indeed, rat platelets, sea urchin sperm and rod outer segments which are enriched in cGMP as well as in enzymes of its metabolism contain very low levels or are devoid of this protein kinase (3,4,6,8). On the other hand, when clearly present this enzyme seems to have a restrictive substrate specificity (18). Furthermore,results by Lincoln (19) have shown that an increase in cGMP levels may not necessarily result in activation of the protein kinase. Therefore alternative mechanisms for cGMP action should be considered. The present work demonstrates that, in addition to the cGMP-dependent protein kinase, a variety of rat tissues contain another type of cGMP binding protein, the cGBPP. The widespread occurrence and the higher levels of this protein in tissues enriched in cGMP as well as its high affinity and high specificity for this nucleotide (3,4) are consistent with the hypothesis that it might mediate a part of the physiological effects of cGMP. Goldberg et coll. (1,20) have previously suggested that the hydrolysis itself of cyclic nucleotides catalyzed by phosphodiesterase regulates cellular processes. It is tempting to imagine that the cGMP binding activity studied here is involved in such a function of the cyclic GMP phosphodiesterase.

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