EFFECTS OF CYCLIC GMP UPON DNA BINDING BY A CALF THYMUS NUCLEAR PROTEIN FRACTION

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Summary. Proteins extracted from calf thymus nuclei with 2 M NaCl and separated by Bio-Rex 70 column chromatography were analyzed for their ability to bind to cyclic nucleotides and to calf thymus DNA. Several nuclear protein fractions contain both cyclic GMP-binding and DNA-binding activities. One protein fraction binds cyclic GMP with an apparent high affinity, as determined by equilibrium dialysis. At low concentrations (10^{-6}M - 10^{-9}M) cyclic GMP slightly enhances the ability of proteins in this fraction to bind ^{125}I-labelled DNA, while at concentrations >10^{-5}M the cyclic nucleotide is strongly inhibitory to DNA binding.

Cyclic GMP (guanosine 3',5'-monophosphate) has been implicated as a possible cellular regulatory agent in a number of animal species and tissues (1, 2). It has been hypothesized that in mammalian cells cyclic GMP represents a mitogenic signal regulating certain events essential to the process of induction of cell proliferation (3). In lymphocytes cyclic GMP has been implicated in the stimulation of both nuclear acidic protein phosphorylation (4) and RNA polymerase I activity (5,6) which occur early upon induction of proliferation by mitogens. In the present report, we provide evidence that cyclic GMP modifies *in vitro* DNA binding by a calf thymus nuclear protein fraction

Materials and Methods. Calf thymus nuclei (10-25g, wet weight) were prepared as described previously (7) and dissociated by incubation for 2 hrs at 4° in 10 volumes of a solution with final concentrations of 2 M NaCl, 1mM EDTA, 1mM dithiothreitol (DTT), 30% glycerol and 0.1 M Tris-Cl, pH 8.0. After centrifugation for 14 hrs at 100,000 x g, the supernatant solution was dialyzed against a solution of 0.05 M NaCl, 1mM EDTA, 1mM DTT, 30% glycerol and 0.01 M Tris-Cl, pH 7.4 containing 100 mg/l of Norit SG-Extra activated charcoal, and centrifuged for 20 min at 20,000 x g to remove insoluble material. Proteins in 60 ml were applied to a Bio-Rex 70 column (7 cm x 3.5 cm diam) in the 0.05 M NaCl buffer and eluted with a linear NaCl gradient (0.05 M to 1.5 M NaCl) in the same buffer.

Eluted fractions were assayed for proteins capable of binding either [^{3}H]cyclic AMP or [^{3}H]cyclic GMP using a filter retention assay based upon that described by Gilman (8) for measuring binding of cyclic AMP to protein. La-

belled cyclic nucleotide (New England Nuclear,Boston,Mass.; [^{3}H]cyclic AMP: 33 Ci/mmole; [^{3}H]cyclic GMP: 3.4 Ci/mmole) was incubated at 1.2 x 10^{-7}M with the protein to be assayed in 2.0 ml of a solution containing 20 mM Mg-acetate, 2mM $CaCl_2$, 1mM 2-mercaptoethanol, 5 x 10^{-5}M papaverine, 10% glycerol and 50mM Na-acetate, pH 6.0. In assays for cyclic GMP binding, unlabelled 5'-GMP and 2', 3'-GMP were each present in the assay mixture at 5 x 10^{-5}M to reduce non-specific binding of the labelled cyclic nucleotide. In assays for cyclic AMP binding, unlabelled 5'-AMP and 2',3'-AMP were similarly present in the assay mixture. After incubation for 10 min at 30°, samples were filtered through Millipore HA filters (0.45 μ pore size), and the filters were washed once with 2 ml of assay buffer, with papaverine and nucleotides omitted, and assayed for radioactivity by scintillation spectrometry.

Equilibrium dialysis was employed to obtain quantitative measurements of cyclic GMP-binding by specific nuclear protein fractions. Protein fractions from the Bio-Rex column (1.0 ml; 500μg of protein) were dialyzed for 60 hrs at 0° against 250 ml of solution containing 5 x 10^{-5}M papaverine, 3mM 2-mercaptoethanol, 1mM $CaCl_2$, 10% glycerol, 50 mM Na-acetate, 10 mM Mg-acetate, pH 6.0, and varying concentrations of [^{3}H] cyclic GMP (3.4 Ci/mmole;New England Nuclear). Aliquots of the dialyzed protein solution and the dialysis buffer were assayed for radioactivity by scintillation spectrometry. Values presented for binding of [^{3}H] cyclic GMP have been corrected for binding obtained in the presence of excess unlabelled cyclic GMP.

The ability of nuclear proteins to bind DNA was measured using a filter retention assay similar to that developed for measuring binding of the lac repressor to the lac operator (9). Heat-denatured calf thymus DNA was labelled with ^{125}I (New England Nuclear) by the procedure of Commerford (10) and used at a specific activity of 3-10 x 10^3 cpm/μg DNA. Proteins (0.1μg - 50 μg) were incubated for 30 min at 25° with [^{125}I]DNA (20 μg) in 2.0 ml of a solution containing 5 x 10^{-5}M papaverine, 0.1mM EDTA, 0.1mM DTT, 50 μg/ml BSA, 5% dimethyl sulfoxide, 0.02 M Tris-acetate, pH 6.9, and various concentrations of cyclic nucleotides. Upon filtration of the incubation mixture through nitrocellulose filters as described (11), DNA-protein complexes are retained and assayed quantitatively by scintillation spectrometry.

Results and Discussion.

Calf thymus nuclear proteins were extracted in 2 M NaCl and fractionated by ion-exchange chromatography on Bio-Rex 70. Each fraction was tested for its affinity for cyclic nucleotides. It can be seen from Fig. 1 that several peaks of cyclic GMP-binding activity are eluted from the Bio-Rex column over a range of NaCl concentrations from 0.25 M to 1.2 M. In contrast, > 90% of the cyclic AMP-binding activity is eluted at 0.05 - 0.1 M NaCl, in the column runoff fraction containing the nuclear acidic proteins. Of all fractions in which cyclic GMP-binding activity is detected, six peaks (numbered I-VI in Fig. 1) contain binding activity which is reduced by addition of unlabelled cyclic GMP to the binding assay mixture. Peak I elutes with the nuclear acidic proteins and includes the peak of highest cyclic AMP-binding activity. This acidic protein fraction also contains all of the cyclic AMP-dependent histone H1 kinase activi-

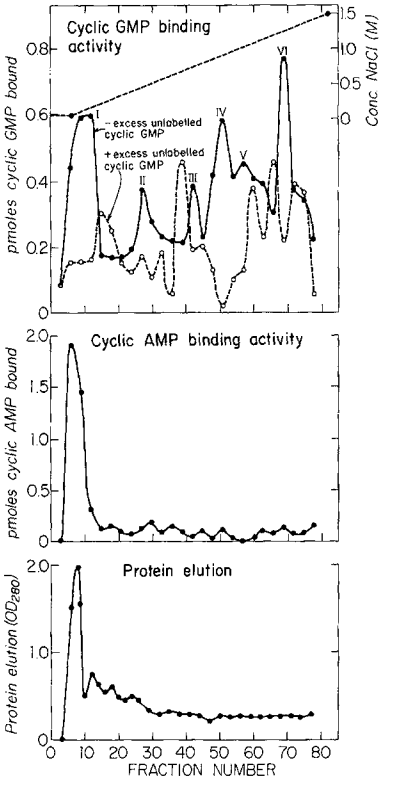

Fig. 1. Binding of [^{3}H]cyclic nucleotides to nuclear protein fractions separated by Bio-Rex 70 column chromatography. Proteins were extracted from calf thymus nuclei with 2M NaCl and subjected to chromatography as described in the text. 10ml fractions were collected. 0.5ml aliquots from every third fraction were incubated with 1.2 x 10^{-7}M [^{3}H]cyclic GMP or [^{3}H]cyclic AMP, and cyclic nucleotide binding to protein was measured by the filter-binding method described in the text. Cyclic GMP-binding activity is depicted in the top panel as measured in either the presence or absence of added 10^{-5}M unlabelled cyclic GMP. Binding of [^{3}H]cyclic AMP, presented in the center panel, was reduced to about 0.8 pmole/0.5ml aliquot in all column fractions by addition of 10^{-5}M unlabelled cyclic AMP to the binding assay medium. Note that several peaks of cyclic GMP-binding activity do not correspond to peaks of cyclic AMP-binding activity.

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ty (data not shown). Cyclic GMP-binding peaks II-VI do not correspond to major peaks of cyclic AMP-binding activity. Peak II elutes between the nuclear acidic proteins (0.05 - 0.1 M NaCl) and the histones (0.6 - 1.5 M NaCl). Cyclic GMP-binding activity in this region is eluted from the Bio-Rex column with 0.25 - 0.4 M NaCl. It can be seen from Fig. 1 that at the concentrations of cyclic

nucleotides used, proteins in peak II bind about 0.4 pmoles of cyclic GMP as compared to less than 0.2 pmoles of cyclic AMP. Cyclic GMP-binding activity in peak II could be stabilized with 30% glycerol, 0.1 mM DTT and 0.1 mM EDTA and stored at -80° for several weeks. Cyclic GMP-binding peaks III-VI elute with the nuclear protein fractions containing histones. Cyclic GMP-binding activity in these basic protein fractions is variable and unstable, and cannot be detected after 2 days at -80° under conditions by which peak II activity is stabilized.

Proteins in Bio-Rex peak II bind cyclic GMP with an apparent high affinity, as determined by equilibrium dialysis (Fig. 2). Curvature of the Scatchard plot of cyclic GMP binding indicates complexity in the interaction of the cyclic nucleotide with its binding sites and may be due to several possibilities. The observed curvature of the Scatchard plot may indicate the presence of multiple classes of cyclic GMP-binding sites, or of negative cooperativity among cyclic

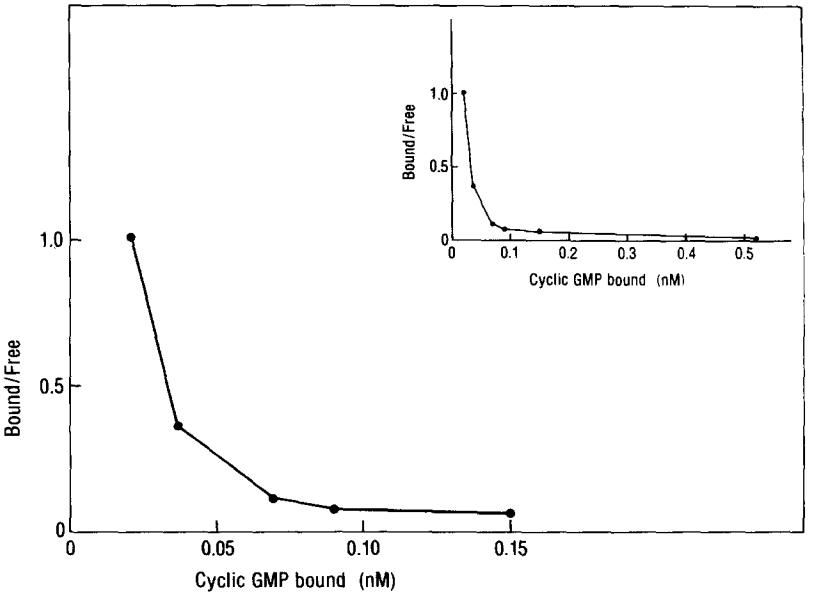

Fig. 2. Scatchard plot of binding of cyclic GMP to Bio-Rex 70 peak II nuclear protein fraction. Nuclear proteins were isolated and separated, and binding of [^{3}H]cyclic GMP was determined by equilibrium dialysis, as described in the text, using concentrations of cyclic GMP ranging from 10^{-8}M to 5 x 10^{-11}M and 1.0 ml protein fractions containing 500 µg of protein.

GMP-binding sites in this fraction. Estimates of apparent equilibrium dissociation constants for binding of cyclic GMP to the nuclear protein fraction, based on reciprocal slopes of curve segments shown in the inset to Fig. 2 range from about 6×10^{-9}M to 2.5×10^{-11}M. This high affinity of cyclic GMP binding suggests that the binding protein(s) involved are not likely to be guanyl cyclase or phosphodiesterase enzymes, which are known to have much lower affinities for the cyclic nucleotide (2). Further experiments are necessary to determine whether cyclic GMP-binding proteins observed in the present study bind nucleotides other than cyclic GMP.

In the presence of cyclic GMP, DNA binding of proteins in Bio-Rex peak II is altered (Fig. 3). Proteins in the peak II fraction bind labelled calf thymus DNA in the absence of added cyclic nucleotide. Low concentrations (10^{-6}M -

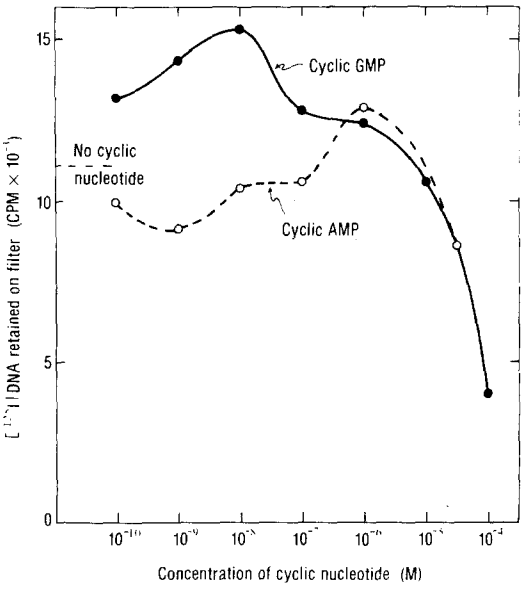

Fig. 3. Effects of cyclic nucleotides upon ability of Bio-Rex 70 peak II nuclear proteins to bind ^{125}I-labelled calf thymus DNA. Protein (10 µg) was preincubated with the cyclic nucleotide for 5 min at 25° before addition of labelled DNA (20 µg; 4400 cpm/µg). Incubation was carried out, and DNA-protein complexes were assayed as described in the text. Values for retention of labelled DNA in the absence of added protein (600-650 cpm) have been substracted so that values presented represent binding of protein to DNA. Standard errors of triplicate measurements were always <5% of the average DNA binding observed.

10^{-10}M) of cyclic GMP stimulate binding of this protein fraction to DNA. In different experiments DNA binding was increased 25-50% and was maximal at 10^{-8}M-10^{-9}M cyclic GMP. High concentrations of cyclic GMP (>10^{-5}M) inhibit DNA binding. It can be seen that 10^{-4}M cyclic GMP inhibits DNA binding as much as 60% (Fig. 3). Cyclic AMP at low concentrations has a slight inhibitory effect upon DNA binding by this nuclear protein fraction, and appears to parallel effects of cyclic GMP at concentrations >10^{-5}M (Fig.3). Fig. 4 depicts the ability of cyclic GMP to modify binding of peak II proteins to DNA at different concentrations of added protein. It can be seen that at protein concentrations less than 10 µg/2 ml, 10^{-4}M cyclic GMP inhibits DNA binding by more than 60% while 10^{-8}M cyclic GMP stimulates DNA binding 1.3-fold to 2-fold.

All cyclic GMP-binding peaks from the Bio-Rex 70 column possess DNA-binding activity. Peak II is the only fraction tested which exhibits enhanced DNA binding in the presence of cyclic GMP. DNA binding by peaks III and IV is inhibited by cyclic GMP, even at concentrations as low as 10^{-9}M. Cyclic GMP-binding activity and effects of cyclic GMP on DNA binding are not observed when

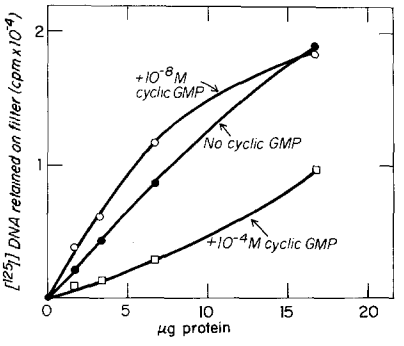

Fig. 4. Effects of 10^{-8}M or 10^{-4}M cyclic GMP upon binding of labelled calf thymus DNA by different concentrations of Bio-Rex 70 peak II nuclear protein. Protein was preincubated in the presence or absence of cyclic nucleotide for 5 min at 25° before addition of labelled DNA (20 µg; 3950 cpm/µg). Incubation was carried out and DNA-protein complexes were assayed as described in the text. Note that 10^{-8}M cyclic GMP enhances binding of this nuclear protein fraction to DNA while 10^{-4}M cyclic GMP strongly inhibits DNA-binding.

crude nuclear proteins are assayed prior to fractionation on the Bio-Rex 70 column.

The functional role of nuclear cyclic GMP-binding proteins is presently not known. The finding that these proteins bind cyclic GMP at 10^{-9}M - 10^{-11} M suggests that this binding could serve some regulatory purpose, since cyclic GMP is present in resting lymphocytes at about 10^{-8}M - 10^{-9}M (3). The nuclear cyclic GMP-binding proteins from calf thymus may be similar to cyclic GMP-binding proteins found associated with RNA polymerase fractions in rat mammary gland nuclear extracts (12). Cyclic GMP has been observed to enhance activity of RNA polymerase I from human lymphocyte nuclei maximally at 10^{-8}M - 10^{-9}M and to inhibit activity at higher concentrations (5,6). It should be noted that in E. coli effects of cyclic nucleotides on RNA synthesis are mediated by binding of a cyclic nucleotide receptor protein to DNA (13,14). The possibility must also be considered that nuclear cyclic GMP-binding proteins include subunits of cyclic GMP-dependent protein kinases. Cyclic GMP-dependent protein kinases have been extracted from mammalian tissues (15,16). This consideration is relevant in view of recent results indicating that cyclic AMP-dependent protein kinase can bind to DNA (11). Cyclic GMP-binding proteins have previously been found in malignant lymphoid tissue (17), and cyclic GMP-binding proteins which bind to DNA have been extracted from calf thymus nuclei (18). The present results are consistent with the possibility that cyclic GMP acts in the cell nucleus early in the induction of lymphocyte proliferation by mitogens.

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References.

1. Hardman, J.G., Beavo, J.A., Gray, J.P., Chrisman, T.D., Patterson, W.D., and Sutherland, E.W. (1971) Annals N.Y. Acad. Sci.185, 27-35.
2. Goldberg, N.D., O'Dea, R.F., and Haddox, M.K. (1973) in Adv. in Cyclic Nucleotide Res. Vol. 3, P. Greengard and G.A. Robison, eds., pp. 155-223, Raven Press, New York.

3. Hadden, J.W., Hadden, E.M., Haddox, M.K., and Goldberg, N.D. (1972) Proc. Nat. Acad. Sci. (U.S.A.) 69, 3024-3027.
4. Johnson, E.M. and Hadden, J.W. (1975) Science 187, 1198-1200.
5. Johnson, L.D., Goldberg, N.D. and Hadden, J.W. (1974) in Adv. in Cyclic Nucleotide Res. Vol. 5, P. Greengard and G.A. Robison, eds., pp. 821-822, Raven Press, New York.
6. Hadden, J.W., Johnson, E.M., Hadden, E.M., Coffey, R.G., and Johnson, L.D. (1975) "Cyclic GMP and Lymphocyte Activation", in Ninth Leukocyte Culture Conference, A.S. Rosenthal, ed.,pp. 359-389, Academic Press, New York.
7. Allfrey, V.G., Inoue, A., Karn, J., Johnson, E.M., and Vidali, G. (1974) Cold Spring Harbor Symposium on Quantitative Biology XXXVIII, 785-801.
8. Gilman, A.G. (1972) in Adv. in Cyclic Nucleotide Res. Vol. 2, P. Greengard, R. Paoletti, and G.A. Robison, eds., pp. 9-24, Raven Press, New York.
9. Riggs., A.D., Suzuki, H., and Bourgeois, S. (1970) J. Mol. Biol. 48, 67-83.
10. Commerford, S.L. (1971) Biochemistry 10, 1993-2000.
11. Johnson, E.M., Hadden, J.W., Inoue, A., and Allfrey, V.G. (1975) Biochemistry, in press.
12. Anderson, K.M., Mendelson, I.S., and Guzik, G. (1975) Biochim. Biophys. Acta 383, 56-66.
13. Riggs, A.D., Reiness, G., and Zubay, G. (1971) Proc. Nat. Acad. Sci. (U.S.A.) 68, 1222-1225.
14. Anderson, W.B., Schneider, A.B., Emmer, M., Perlman, R.L., and Pastan, I. (1971) J. Biol. Chem. 246, 5929-5937.
15. Greengard, P., and Kuo, J.F. (1970) in Role of Cyclic AMP in Cell Function, P. Greengard and E. Costa, eds. pp. 286-306, Raven Press, New York.
16. Kuo, J.F. (1974) Proc. Nat. Acad. Sci. (U.S.A.) 71, 4037-4041.
17. Masaracchia, R., and Walsh, D.A. (1974) Fed. Proc. 33, 1324.
18. Allfrey, V.G., Inoue, A., Karn, J., Johnson, E.M., Good, R.A., and Hadden, J.W. (1975) in The Structure and Function of Chromatin, Ciba Foundation Symposium 28, pp. 199-218, Elsevier, Amsterdam.