# ROLE OF 3', 5'-CYCLIC AMP IN THE CONTROL OF NUCLEAR PROTEIN KINASE ACTIVITY

Lora E. Rikans and Raymond W. Ruddon[1]
Department of Pharmacology
The University of Michigan Medical School
Ann Arbor, Michigan 48104

Received June 7,1973

## SUMMARY

The role of cAMP in the regulation of nuclear protein kinase activity was investigated. Acidic nuclear proteins prepared from rat liver nuclei were separated by phosphocellulose chromatography into four peaks of protein kinase activity and two peaks of cAMP-binding activity. A fraction which bound cAMP also inhibited the most active nuclear protein kinase, K IV, and the inhibition was diminished in the presence of 5 $\mu$M cAMP. Further support for the regulation of nuclear kinases by cAMP was obtained using a regulatory subunit prepared from rabbit muscle protein kinase. The muscle regulatory subunit markedly inhibited liver nuclear kinase activities. The addition of cAMP partially restored the activities.

Nuclear phosphoproteins have been implicated in the specific control of gene transcription in mammalian cells. Both histone and nonhistone nuclear proteins are phosphorylated, and the degree of phosphorylation seems to determine their ability to interact with DNA and influence transcription (1-6). Thus, to understand the control of gene readout in mammalian systems it is important to understand the nature of the protein kinases involved in the phosphorylation of nuclear proteins.

Multiple protein kinase activities have recently been demonstrated in rat liver nuclei (7-10). These investigations all failed to demonstrate an activation of the nuclear protein kinases by cyclic adenosine-3', 5' monophosphate (cAMP). Yet the in vivo phosphorylation of rat liver nonhistone proteins was stimulated about 2-fold by $N^6, O^{2'}$-dibutyryl cyclic AMP (11). Evidence from several laboratories supports a common mechanism of activation of protein kinases from several different tissues

[1] Research support from NIH Grant CA-12765.

by cAMP (12-16). The mechanism proposes that inactive protein kinase holoenzyme can be dissociated into an active catalytic subunit and a cAMP-binding regulatory subunit in the presence of cAMP. This study was undertaken to investigate the possibility that nuclear kinases may be dissociated catalytic subunits which have become separated from their regulatory subunits during preparation. Its purpose was to determine whether or not cAMP plays a role in the control of nuclear protein kinase activity.

## METHODS

The methods used for the preparation of acidic nuclear proteins (ANP) from purified rat liver nuclei, and the separation of nuclear kinase activities by phosphocellulose chromatography were described in an earlier report (8). ANP (15-20 mg) was placed on a phosphocellulose (Whatman P-11) column, and elution was begun with 125 ml of the equilibrating buffer (0.05 M Tris-HCl, pH 7.5-0.4 M NaCl) followed by 175 ml of 0.05 M Tris-HCl, pH 8.1-0.6 M NaCl. The column fractions were assayed for protein kinase activity by measuring the incorporation of $^{32}P$ from ATP-$\gamma$-$^{32}P$ into acid-precipitable material as described earlier (8).

The cAMP-binding activity of the column fractions was measured by a modification of the membrane filtration technique of Tao (17). The binding mixture contained (mM), in a final volume of 0.5 ml: Tris-HCl (pH 8.0), 100; $\beta$-mercaptoethanol, 5; $MgCl_2$, 4; $^3H$-cAMP, 0.0005 (5,000 cpm/pmole); and column eluate. The mixture was incubated at 37° for 3 min, transferred to an ice bath, and diluted with 5 ml of cold buffer (buffer B) containing (mM): Tris-HCl (pH 8.0), 100; $\beta$-mercaptoethanol, 5; and $MgCl_2$, 4. The binding mixture was then filtered through a nitrocellulose filter (0.45 $\mu$ pore size). After washing 3 times with cold buffer B, the filter was dried, suspended in toluene scintillator, and counted in a Packard Tri-Carb liquid scintillation spectrometer.

A preparation containing the regulatory subunit of rabbit muscle protein kinase was made by dissociating the kinase into its catalytic and regulatory subunits and selectively inactivating the catalytic subunit. Rabbit muscle protein kinase was generously provided by Dr. John Voorhees, Department of Dermatology, The University of Michigan Medical School. The protein kinase preparation was dialyzed 3 hrs against a solution of

10 mM Tris-HCl (pH 8.0) - 5 mM $\beta$-mercaptoethanol, and then made $5 \times 10^{-5}$ M with respect to 3', 5'-cyclic GMP prior to incubating at 53° C for 5 min to inactivate the kinase activity (14). After transferring to an ice bath, the preparation was dialyzed overnight against a buffer containing (mM): Tris-HCl (pH 8.0), 10; $\beta$-mercaptoethanol, 5; and $MgCl_2$, 4. This treatment resulted in a regulatory subunit preparation (RS) which actively bound cAMP (46 $\mu$g retained 1600 cpm in the cAMP binding assay) but had negligible protein kinase activity.

## RESULTS AND DISCUSSION

The elution profile of protein kinase and cAMP-binding activities is illustrated in Figure 1. The cAMP-binding peaks, A and B, were

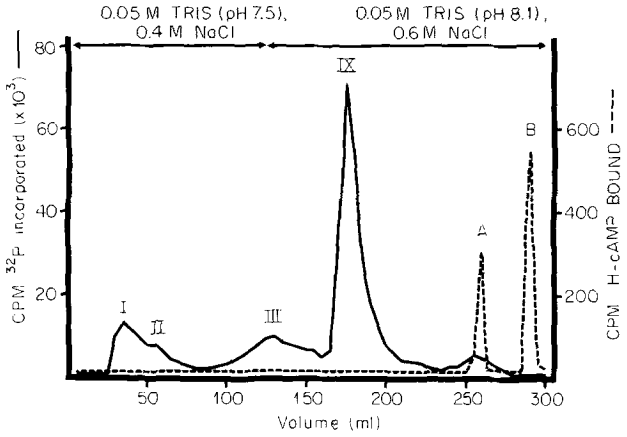


Fig. 1. Separation of Protein Kinase and cAMP-Binding Activities by Phosphocellulose Chromatography.
Fifteen mg of ANP was applied to a phosphocellulose column (0.9 x 26 cm) equilibrated with 0.05 M Tris-HCl (pH 7.5) - 0.4 M NaCl. After 25 five-ml fractions were collected, the buffer was changed to 0.05 M Tris-HCl (pH 8.1) - 0.6 M NaCl. The samples were assayed for kinase activity (——) and cAMP-binding (----) as described in the Methods.

eluted well after the four kinase activity peaks. The elution of the cAMP-binding peaks was variable. In 4 out of 7 experiments the peaks appeared in the positions shown in Figure 1. In some experiments, however, the cAMP-binding activity appeared as several smaller peaks. The variability

was attributed to the lability of these fractions since cAMP-binding activity was observed to be significantly decreased in some fractions which were allowed to stand at 4° overnight. As reported in a previous publication from this laboratory (8), the majority of the protein found in the effluent appeared as a large peak coinciding with kinase I (K I). Kinases II and III were unstable and were not studied extensively. The major activity peaks, kinase IV (K IV) and K I were capable of phosphorylating ANP, but casein was a more active substrate. Unlike the soluble rat liver protein kinases (15, 18), the nuclear kinases studied in our experiments do not actively phosphorylate histone (19). Furthermore, several reports have indicated that nuclear kinases are not stimulated by cAMP (7-10). The observation that phosphocellulose chromatography of ANP separated cAMP-binding fractions from the protein kinase activities provides an explanation for the lack of activation of these nuclear kinases by cAMP.

Experiments were conducted to see if the cAMP-binding fractions from the column could inhibit the activity of nuclear protein kinases, K I and K IV. Neither peak A nor B inhibited the activity of K I (phosphorylation of ANP which co-chromatographed with K I). The activity of K IV, however, was inhibited by the addition of peak B (Table 1). The inhibition was partially reversed by the addition of 5 $\mu$M cAMP to the incubation mixture. These findings are consistent with the hypothesis that nuclear kinases may be catalytic subunits separated during preparation from their inhibitory, or regulatory, subunits. The small degree of inhibition observed after the addition of the binding peaks may be due to the fact that the protein concentration (less than 10 $\mu$g/ml) as well as the cAMP-binding activity of peaks A and B was low (500 cpm represents 0.1 pmole cAMP bound). In addition, the cAMP-binding material was labile. More concentrated preparations of the cAMP-binding materials are necessary to adequately determine whether or not they are the proposed regulatory proteins.

The regulatory subunits of cAMP-dependent protein kinases are able to interact with and inhibit the catalytic subunits from different tissues and different species (20, 21). Therefore, the possibility that a regulatory subunit may modify the activity of nuclear kinases was further tested by using rabbit muscle regulatory subunit (RS). The RS, prepared from rabbit muscle protein kinase as described in the Methods, consistently inhibited

TABLE 1

Effect of cAMP-Binding Fractions on
the Activity of Nuclear Protein Kinase, K IV

| Additions to Complete System[a] | cpm $^{32}$P Incorporated | |
|---|---|---|
| | Exp. 1 | Exp. 2 |
| None | 32,199 | 27,151 |
| cAMP | 27,469 | 27,176 |
| Peak A | 30,573 | 28,718 |
| Peak A + cAMP | 29,475 | 27,046 |
| Peak B | 24,832 | 22,291 |
| Peak B + cAMP | 27,682 | 24,298 |
| 10 μg Albumin | 31,200 | |

[a]The complete system was incubated for 10 min at 30° in a total volume of 1.0 ml, containing (mM): Tris-HCl (pH 8.0), 50; β-mercaptoethanol, 5; $Mg(CH_3COO)_2$, 8; NaCl, 100; $^{32}$P-ATP, 0.01 (0.4 μCi); 100 μg of casein; and 25 μg of dialyzed K IV. The incorporation of $^{32}$P was linear for 10 min under these conditions. After stopping the reaction with 10% trichloroacetic acid containing 0.04 M $Na_4P_2O_7$ and 1.0 mM ATP, the samples were filtered, washed and counted as previously described (8). Samples were run in triplicate. The concentration of cAMP was $5 \times 10^{-6}$ M. Column fractions containing Peak A or Peak B were dialyzed overnight against 0.05 M Tris-HCl (pH 8.0), and 0.2 to 0.4 ml were added to the reaction. Bovine serum albumin (10 μg) was added as an additional control in Exp. 1.

the activities of both K I and K IV (Table 2). The inhibition was reversed in the presence of 5 μM cAMP. The most dramatic inhibition was obtained with freshly prepared RS (Exp. 1, Table 2). Freezing the RS for two weeks resulted in a 50% decrease in cAMP-binding activity and a large decrease in ability to inhibit kinase activity. Other investigators have also reported that cAMP-binding regulatory subunits are labile and that they are readily degraded in vitro (21-23).

The inhibition of kinase activity by the addition of cAMP alone was an unexpected result. High concentrations of cAMP (50-500 μM) have been reported to inhibit the activity of protein kinases from a variety of tissues, probably by competing with ATP at the catalytic site of the enzyme (24). It is possible that the partially purified nuclear kinases used in these experi-

TABLE 2

Effect of Rabbit Muscle Regulatory Subunit
on the Activity of Nuclear Protein Kinases

| Kinase | Additions[a] | cpm $^{32}$P Incorporated | | |
|---|---|---|---|---|
| | | Exp. 1 | Exp. 2 | Exp. 3 |
| K IV | None | 11,055 | | 10,132 |
| | cAMP | 3,068 | | 3,773 |
| | RS | 4,003 | | 8,065 |
| | RS + cAMP | 7,973 | | 8,709 |
| K I | None | | 414 | 869 |
| | cAMP | | 342 | 74 |
| | RS | | 276 | 692 |
| | RS + cAMP | | 533 | 766 |

[a]The incubation mixture (1.0 ml) contained (mM): Tris-HCl (pH 8.0), 100; $Mg(CH_3COO)_2$, 8; NaCl, 80-120; and $^{32}$P-ATP, 0.01 (0.4 $\mu$Ci). Casein (100 $\mu$g) was the substrate for K IV (25 $\mu$g), and the endogenous ANP in the K I fraction (100 $\mu$g) was the substrate for K I. Assay conditions were as described in Table 1. The amount of rabbit muscle regulatory subunit (RS) added was 9-10 $\mu$g; the concentration of cAMP was $5 \times 10^{-6}$ M.

ments are more sensitive to the inhibitory effects of cAMP. In this respect, Johnson and Allfrey (11) reported maximum stimulation of phosphorylation of ANP in rat liver nuclei at 5 $\mu$M cAMP, while higher concentrations of cAMP inhibited the stimulatory effect. In our experiments inhibition by cAMP was less pronounced in the presence of $\beta$-mercaptoethanol, in solutions of lower ionic strength, or when the kinase was dialyzed extensively, as indicated by the results in Table 1 versus those in Table 2.

These studies provide evidence that 1) rat liver ANP contains cAMP-binding material as well as protein kinase activity, 2) that nuclear protein kinase activity can be reversibly inhibited by the cAMP-binding fraction, and 3) that nuclear kinase activity can be reversibly inhibited by the regulatory subunit from rabbit muscle cAMP-dependent protein kinase. The evidence is consistent with a model in which the activity of nuclear kinases is controlled by a cAMP-binding subunit.

REFERENCES

1. Kleinsmith, L. J., Allfrey, V. G. and Mirsky, A. E., Proc. Nat'l. Acad. Sci. U. S., 55: 1182 (1966).

2. Kleinsmith, L. J., Allfrey, V. G., and Mirsky, A. E., Science 154: 780 (1966).

3. Teng, C. S., Teng, C. T., and Allfrey, V. G., J. Biol. Chem. 246: 3597 (1971).

4. Langan, T. A., Science 162: 579 (1968).

5. Gurley, L. R., Walters, R. A. and Tobey, R. A., Arch. Biochem. Biophys. 154: 212 (1973).

6. Fontana, J. A. and Lovenberg, W., Proc. Nat'l. Acad. Sci. U. S., 70: 755 (1973).

7. Takeda, M., Yamamura, H. and Ohga, Y., Biochem. Biophys. Res. Commun. 42: 103 (1971).

8. Ruddon, R. W. and Anderson, S. L., Biochem. Biophys. Res. Commun. 46: 1499 (1972).

9. Desjardins, P. R., Lue, P. F., Liew, C. C. and Gornall, A. G., Can. J. Biochem. 50: 1249 (1972).

10. Kamiyama, M. and Dastugue, B., Biochem. Biophys. Res. Commun. 44: 29 (1971).

11. Johnson, E. M. and Allfrey, V. G., Arch. Biochem. and Biophys. 152: 786 (1972).

12. Brostrom, M. A., Reimann, E. M., Walsh, D. A. and Krebs, E. G., Advan. Enzyme Regul. 8: 191 (1970).

13. Gill, G. N. and Garren, L. D., Biochem. Biophys. Res. Commun. 39: 335 (1970).

14. Tao, M., Salas, M. L., and Lipmann, F., Proc. Nat'l. Acad. Sci. U. S., 67: 408 (1970).

15. Kumon, A., Yamamura, H., and Nishizuka, Y., Biochem. Biophys. Res. Commun., 41: 1290 (1970).

16. Miyamoto, E., Petzold, G. L., Harris, J. S. and Greengard, P., Biochem. Biophys. Res. Commun. 44: 305 (1971).

17. Tao, M., Arch. Biochem. Biophys., 143: 151 (1971).

18. Yamamura, H., Takeda, M., Kumon, A., and Nishizuka, Y., Biochem., Biophys. Res. Commun. 40: 675 (1970).

19. Ruddon, R. W. and Rikans, L. E., unpublished data.

20. Miyamoto, E., Petzold, G. L., Kuo, J. F. and Greengard, P., J. Biol. Chem. 248: 179 (1973).

21. Yamamura, H., Nishiyama, K., Shimomura, R. and Nishizuka, Y., Biochem. 12: 856 (1973).

22. Tao, M., Biochem. Biophys, Res. Commun. 46: 56 (1972).

23. Corbin, J. D., Brostrom, C. O., King, C. A. and Krebs, E. G., J. Biol. Chem. 247: 7790 (1972).

24. Donnelly, T. E., Jr., Kuo, J. F., Miyamoto, E. and Greengard, P., J. Biol. Chem. 248: 199 (1973).