# A PROTEIN KINASE ACTIVITY FROM RAT CEREBELLUM STIMULATED BY GUANOSINE-3':5'-MONOPHOSPHATE §

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Summary

The influence of cyclic nucleotides on a protein kinase activity present in the 50,000 x g supernatant of rat cerebellum has been studied. cGMP ($3 \times 10^{-7}$M) increased histone phosphorylation by about 90 %. The apparent $K_a$ for cGMP was $3.6 \times 10^{-8}$M. cAMP had a similar effect, but higher concentrations ($10^{-5}$M) were required to obtain a comparable stimulation, the apparent $K_a$ being $9 \times 10^{-7}$M. In a phosphate free buffer cGMP had only a slight effect on the rate of phosphorylation, whereas cAMP stimulated the reaction by 100 %. The addition of phosphate resulted in a twofold increase of the reaction stimulated by cGMP and in a marked decrease of the phosphorylation stimulated by cAMP. In addition, different pH optima for the two reactions were observed. It is concluded that in the rat cerebellum a cGMP stimulated protein kinase exists.

In the cerebellum and in several other mammalian tissues the levels of cGMP[§§] can be influenced by substances affecting cholinergic mechanismen (1-4). The cAMP concentration remains unchanged under these conditions. This supports the concept of independent roles for cGMP and cAMP (5). However, the interpretation of these observations is difficult, because so far no system is known which is regulated by cGMP in mammalian tissues. In analogy to what has been found for cAMP one part of such a system could be a protein kinase regulated by cGMP. To examine such a possibility we studied the ability of cGMP and cAMP to stimulate the phosphorylation of histone by a protein kinase preparation obtained from rat cerebellum.

## Materials and Methods

ATP, cAMP, cGMP, 5'-AMP and 5'-GMP were purchased from Boehringer Mannheim GmbH. $\gamma$-$^{32}$P-ATP and $\gamma$-$^{32}$P-GTP were obtained from Amersham Buchler, 8-$^{3}$H

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§§Abbreviations used: cGMP, Guanosine-3':5'-monophosphate; 2':3'-GMP, Guanosine-2':3'-monophosphate; cAMP, Adenosine-3':5'-monophosphate; cNMP, Nucleotide-3':5'-monophosphate; GP, Sodium glycerophosphate; TES, N-tris-(hydroxymethyl)methyl-2-aminopropan-sulfonic acid; PEI-cellulose, Polyethylenimine-cellulose; ANDA, 7-amino-1,3-naphthalene-disulfonic acid.



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cAMP (spec.act. 24.3 C/mmole) from NEN Chemicals and 8-$^{3}$H-cGMP (spec.act. 24.7 C/mmole) from ICN Corp.; they were examined by TLC on PEI-cellulose and found to be free of radioactive impurities. The tritiated cyclic nucleotides were rechromatographed before use. Histone II A and 2':3'-GMP were obtained from Sigma Chem. Corp., all other histone types from Worthington Biochemicl Corp.

Tissue preparation: Male Wistar rats (150-250 gm) were decapitated. The cerebellum was quickly removed and rinsed in cold 10 mM Tris/HCl buffer (pH 7.5) containing 1 mM dithioerythritol. 3 to 4 cerebella were pooled and homogenized in six volumes of the same buffer in a Potter-Elvehjem glass-teflon homogenizer and centrifuged for 60 min. at 50,000 x g (0-4°C). In all experiments the supernatant was used.

Assay of protein kinase activity: The incubation mixture contained 10 mM magnesium acetate; 2 mM theophylline; 0.3 mM ethylene glycol-bis-(ß-aminoethyl ether)-N,N'-tetraacetic acid; 0.1 mM $\gamma$-$^{32}$P-ATP (containing about 250,000cpm); 40 µg histone II A; with or without 1 µM cGMP or 1 µM cAMP in a total volume of 0.1 ml, and 50 mM GP buffer (pH 7.5). The reaction was started by the addition of aliquots of the 50,000 x g supernatant ( 45-55 µg protein). After incubation for 5 or 10 min. at 30°C the reaction was stopped by adding 50 µl of a 0.63 % bovine serum albumin solution as carrier protein and 0.2 ml of 30 % TCA. Protein-bound $^{32}$P was separated from $\gamma$-$^{32}$P-ATP according to Kuo et al. (6) and the radioactivity was determined by using Cerenkov radiation in 0.1 % ANDA (7) with the addition of 0.2 ml 2N HCl. One unit of protein kinase activity is defined as the amount of enzyme which transfers one pmole of phosphate from ATP to histone in 10 min. at 30°C. Protein was determined according to Lowry et al. (8) with bovine serum albumin as standard. If not otherwise indicated, values reported are the mean of triplicate incubations ± S.E.M.

## Results and Discussion

Histone was phosphorylated only with $\gamma$-$^{32}$P-ATP as phosphate donor; no transfer of phosphate from $\gamma$-$^{32}$P-GTP has been detected.

The phosphorylation of histone was proportional to the amount of enzyme preparation both in the presence and absence of cyclic nucleotides (Fig. 1). In the absence of histone a small phosphorylation of an endogenous substrate was detectable which, however, could not be stimulated by either cGMP or cAMP.

The phosphorylation of the endogenous substrate and of histone required a divalent cation (Table I). Unstimulated phosphorylation occured in the presence of magnesium, zink, manganese, and cobalt, but at different rates. Stimulation by cyclic nucleotides, however, could only be detected in the presence

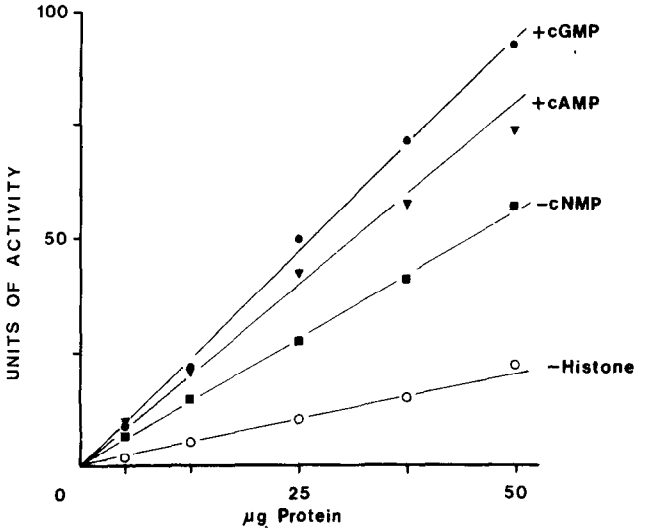


Fig 1 Histone phosphorylation as a function of protein concentration.- Assay conditions were as described under " Methods " except for varying the amount of protein from the 50,000 x g supernatant. The enzyme preparation was dialyzed for four hours against 400 volumes of homogenization buffer.

Table I: Effect of cations on the protein kinase activity. - Assay conditions were as described under " Methods " except for varying kind and amount of cation. In the absence of cation no activity was observed.

| Cation | | Protein kinase activity in the presence of | | | |
|---|---|---|---|---|---|
| | | no histone | histone and | | |
| | | | no cNMP | cGMP | cAMP |
| | | units | | | |
| $Mg^{++}$ | 2.5 mM | 21 ± 0.5 | 40 ± 0.2 | 60 ± 2.0 | 53 ± 1.0 |
| | 10 mM | 25 ± 1.0 | 61 ± 0.5 | 98 ± 2.0 | 78 ± 0.3 |
| $Co^{++}$ | 2.5 mM | 24 ± 0.7 | 53 ± 0.5 | 76 ± 0.3 | 75 ± 2.0 |
| | 10 mM | 20 ± 1.0 | 51 ± 4.0 | 52 ± 1.0 | 56 ± 1.0 |
| $Mn^{++}$ | 2.5 mM | 24 ± 0.4 | 48 ± 0.6 | 55 ± 2.0 | 54 ± 3.0 |
| | 10 mM | 30 ± 1.0 | 58 ± 1.0 | 64 ± 5.0 | 63 ± 2.0 |
| $Zn^{++}$ | 2.5 mM | 9 ± 0.5 | 16 ± 0.3 | 19 ± 1.0 | 18 ± 0.5 |
| | 10 mM | 10 ± 0.3 | 20 ± 0.5 | 23 ± 0.2 | 21 ± 0.5 |
| $Ca^{++}$ | 2.5 mM | 4 ± 0.3 | 5 ± 0.5 | 5 ± 0.2 | 5 ± 0.5 |
| | 10 mM | 4 ± 0.3 | 5 ± 0.2 | 5 ± 0.5 | 5 ± 0.5 |

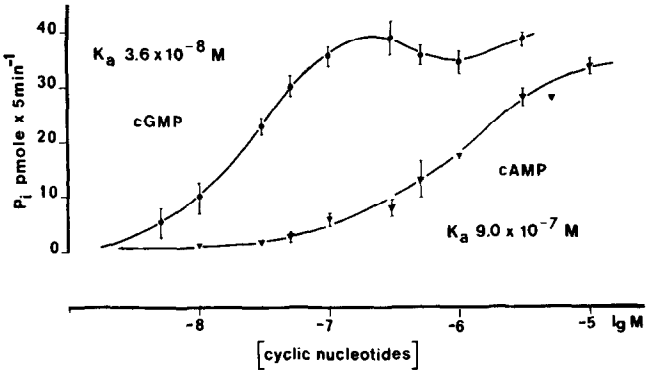


Fig. 2 Protein kinase activity in the presence of varying concentrations of cGMP and cAMP. - Assay conditions were as described under " Methods " except for the variation in the cyclic nucleotide concentration. Incubation time was 5 min. Values are the mean ± S.E.M. for two experiments with two different enzyme preparations. Activity in the absence of cyclic nucleotides has been subtracted and amounted for 58 or 57 pmoles phosphate transferred per 5 min., respectively. Protein concentration was 52 µg per tube for both experiments.

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of magnesium (2.5 or 10 mM) or of cobalt (2.5 mM). Manganese could replace only partially magnesium in the stimulated reactions. This might be due to the stimulatory effect of manganese on the dephosphorylation of histone by a phosphoprotein phosphatase; it has such an action on a phosphoprotein phosphatase from cerebrum (9). In the presence of calcium, the endogenous substrate was phosphorylated at a low rate, but not histone.

Maximal histone phosphorylation was almost identical for both cyclic nucleotides, reaching about 190 % of the unstimulated reaction (Fig.2). However the concentration of cAMP necessary (1 x $10^{-5}$ M) was 30 times higher than that of cGMP (3 x $10^{-7}$ M). From Eadie-Hofstee plots an apparent $K_a$ of 3.6 x $10^{-8}$ M for cGMP and 9 x $10^{-7}$ M for cAMP were obtained. No increase in histone phosphorylation was observed with 5'-GMP, 5'-AMP, and 2':3'-GMP. To exclude the possibility that a phosphodiesterase activity is responsible for the difference in the apparent $K_a$ values, the degradation of tritiated cyclic nucleotides under standard conditions was measured. Following incubation the cyclic nucleotides were separated from degradation products by TLC on PEI-cellulose according to Böhme (10). Between 80 and 90 % of the radioactivity were found in positions corresponding to cGMP or cAMP. At each concentration used, the rate of degradation was identical for the two cyclic nucleotides. Therefore it is concluded that the difference in the apparent $K_a$ values is not due to different degradation rates of the cyclic nucleotides. However, it cannot be decided whether cGMP and cAMP stimulate the same or two separate enzymes.

Table II: Influence of cGMP and cAMP on the phosphorylation of different histone types. - Incubation conditions were as described under " Methods " except for varying the kind of histone. 40 µg of the respective histone was used per tube. Values have been corrected for the activity in the absence of histone, which amounted to 24 ± 0.2 units.

| Histone | Protein kinase activity in the presence of | | |
|---|---|---|---|
| | no cNMP | cGMP | cAMP |
| | units | | |
| Histone II A | 69 ± 2 | 93 ± 2 | 83 ± 1 |
| HLY Mixture | 87 ± 4 | 115 ± 2 | 113 ± 1 |
| Hf3 Arginine rich | 75 ± 1 | 90 ± 0 | 87 ± 7 |
| HA Arginine rich | 61 ± 2 | 69 ± 1 | 73 ± 2 |
| HL Lysine rich | 64 ± 2 | 85 ± 6 | 82 ± 4 |
| Hf2b Slightly Lysine rich | 113 ± 6 | 148 ± 1 | 169 ± 2 |

An attempt to differentiate these two possibilities was made by using various histone types as substrates (Table II), particularly because the cGMP and cAMP dependent protein kinases from Arthropods have different affinities for distinct histone types (11). Among the histones examined the highest rate of phosphorylation was obtained with " slightly lysine rich " histone in the presence of cAMP, whereas cGMP was less effective. The reverse relation was found with histone II A.

The stimulating effect of both cyclic nucleotides was further studied by replacing the GP buffer by TES buffer (Fig.3).In this buffer cAMP enhances phosphorylation by 100 % compared to 40 % in the GP buffer, whereas the stimulation by cGMP is decreased from 100 % to 40 %. The addition of 50 mM phosphate to the TES buffer resulted in phosphorylation rates which were very close to those obtained in the GP buffer. The effect of phosphate depends on the concentration used. At high concentrations (200 mM) all three reactions are inhibited, whereas at lower concentrations the unstimulated and the cAMP stimulated phosphorylation are reduced while the cGMP stimulated reaction is enhanced.

The pH dependence of the stimulated reactions was studied in a Soerensen phosphate buffer (Fig.4). At 33 mM phosphate the cAMP stimulated phosphorylation exhibited two pH optima at pH 6.0 and pH 7.5, respectively. In contrast, the cGMP stimulated reaction had only one pH optimum at pH 7.5. When

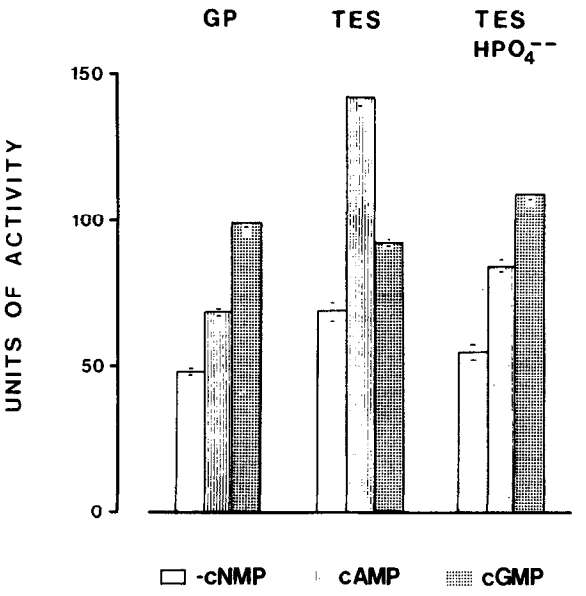


Fig 3 Influence of phosphate on the protein kinase activity. - Assay conditions were as described under " Methods " except for varying the kind of incubation buffer. The concentration of GP, TES, and inorganic phosphate was 50 mM, the pH 7.5.

the phosphate concentration was increased to 66 mM the lower pH optimum for cAMP was no longer detectable. At the higher pH optimum cGMP was more effective than cAMP in stimulating histone phosphorylation which is in accordance with the results mentioned above.

Attempts to separate the protein kinase activity stimulated by cGMP from that stimulated by cAMP were made by ammonium sulfate fractionation at pH 7.5 (Table III). The fraction which precipitated between 40 and 60 % saturation contained about 60 % of the cGMP stimulated enzyme activity; the specific activity was increased. The ratio of the cGMP dependent to independent histone phosphorylation was increased from 2.1 in the crude supernatant to 5.5 in the ammonium sulfate fraction, whereas the same ratio for cAMP was only enhanced from 1.6 to 5.5. Further experimental approaches to purify the cGMP " dependent " enzyme (chromatography on DEAE-cellulose or sepharose-4B-protamine sulfate columns and calcium phosphate gel treatment) resulted in an almost complete loss of the cGMP stimulated activity.

So far cGMP regulated protein kinases have only been found in Arthropods (6,11). For some mammalian tissues protein kinases with relative specificity for cGMP have been mentioned (12). But, since only one concentration of cGMP and cAMP has been compared this report needs further confirmation.

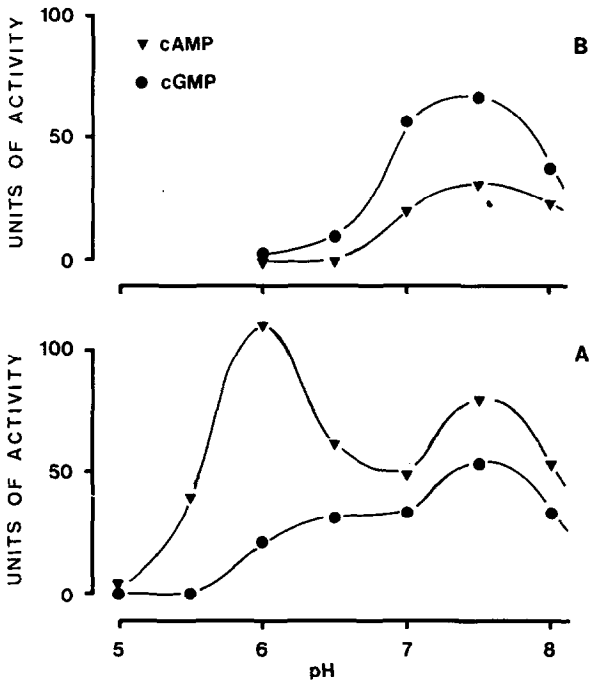


Fig 4 Influence of pH on the stimulated histone phosphorylation. - Assay conditions were as described under " Methods " except for varying the pH of a phosphate buffer (Soerensen), which substituted for the GP buffer. The concentration of phosphate was maintained constant at 33 mM for A and at 66 mM for B, respectively.

Table III: Ammonium sulfate fractionation of the rat cerebellum supernatant. The 40 to 60 % ammonium sulfate fraction was dialyzed over night against the homogenization buffer. The last columns show the ratio of independent to dependent histone phosphorylation for the respective cyclic nucleotide.

| Fraction | Protein kinase activity in the presence of | | | Ratio for | |
|---|---|---|---|---|---|
| | no cNMP | cGMP | cAMP | cGMP | cAMP |
| | units x mg Prot.$^{-1}$ | | | | |
| Supernatant fraction | 987 | 2050 | 1590 | 2.1 | 1.6 |
| 40-60 % $(NH_4)_2SO_4$ fraction | 674 | 3680 | 1680 | 5.5 | 2.5 |

Therefore it is doubtful, whether in all mammalian tissues the effects of cGMP are mediated through a protein kinase. However the data presented indicated that at least in the rat cerebellum either a cGMP " dependent " protein kinase

exists, which can be stimulated also by higher concentrations of cAMP - or that two different protein kinases are present. In accordance with the first possibility the difference in the apparent $K_a$ values is similar to that found for the cGMP dependent protein kinase from lobster tail muscle (6). But the differential influence of phosphate and pH on the stimulation by cGMP and cAMP is in favor of the latter possibility. One may speculate that these enzymes are composed of similar moieties as it has been reported for the cAMP dependent protein kinases (13-16). This would imply the presence of separate catalytic and regulatory subunits. Consequently cGMP and cAMP should stimulate the phosphorylation of different physiological substrates. Since the protein kinases from rat cerebellum phosphorylate quite similar substrates and since it is unknown how many regulatory subunits are bound to a catalytic subunit, a third possibility could be that the enzyme consists of one catalytic but two regulatory subunits, one binding cGMP and one binding cAMP. Further purification of the cGMP stimulated protein kinase and its physiological substrate is necessary to prove one of these possibilities.

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