INHIBITORY EFFECTS OF CYCLIC-AMP DEPENDENT PROTEIN KINASE ON GUANYLATE CYCLASE ACTIVITY IN RAT CEREBELLUM

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1. Introduction

Guanylate cyclase (EC 4.6.1.2), the enzyme responsible for the formation of cyclic GMP from GTP, has been studied in homogenates or both supernatant and particulate fractions of several tissues [1–8].

Recently, various authors suggested that soluble and particulate guanylate cyclase have different kinetic properties and molecular size [9–11], and that the different forms of the enzyme may be independently regulated. Nevertheless, the regulatory mechanisms of guanylate cyclase activity remain unclear, although the importance of Ca^{2+} has been demonstrated in many tissues [12–15].

In the present study we observed an inhibitory effect of 3',5'-cyclic adenosine monophosphate (cyclic AMP) dependent protein kinase (ATP, protein phosphotransferase (EC 2.7.1.37)) on guanylate cyclase activity in rat cerebellar homogenates. A possible regulatory mechanism of guanylate cyclase is proposed.

2. Materials and methods

Cerebella from Sprague-Dawley rats (Charles River) were used for guanylate cyclase preparation. [8-^{3}H] GTP (11 Ci/mmol) and [γ-^{32}H] ATP (16.2 Ci/mmol) were obtained from Radiochemical Centre (Amersham). Unlabeled cyclic GMP, GTP, cyclic AMP, ATP, as well as creatine phosphate and creatine kinase were purchased from Boehringer Mannheim. Calf thymus histone was from Biochemia. DEAE-cellulose (Cellex D) was obtained from BioRad Laboratories.

2.1. *Purification of cAMP-dependent protein kinase*

Cyclic AMP-dependent protein kinase from bovine heart muscle was purified on a DEAE-cellulose column with the method of Kuo et al. [16].

After purification the specificity of the protein kinase for cyclic AMP was examined according to Kuo et al. [16] with 5×10^{-6} M cyclic AMP. The specific activity of the purified protein kinase was 594 and 6300 U/mg protein in the absence and presence of 5×10^{-6} M cAMP respectively. One unit of the enzyme activity is defined as the amount of enzyme able to transfer 1 pmol of ^{32}P from [γ-^{32}P] ATP to recovered histone in 5 min at 30°C in the standard assay system. The purified protein kinase was stored in aliquots at –40°C.

2.2. *Standard guanylate cyclase assay*

Rat cerebella were homogenized in a Potter-Elvehjem teflon glass homogenizer with 10 volumes of 0.25 M sucrose containing 0.02 M Tris–HCl buffer (pH 7.4), 1 mM EDTA and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 105 000 $\times$ *g* for 60 min at 2°C. The supernatant thus obtained was employed as soluble enzyme preparation. The precipitate, once washed, was resuspended in the same volume of buffer used for homogenization. The suspension was employed as particulate enzyme preparation. Guanylate cyclase activity was measured according to the method of Nakazawa and Sano [17] with some modifications. The reaction mixture contained

40–100 μg of enzyme protein, an incubation medium consisting of 60 mM Tris–HCl buffer (pH 7.4), 2 mM cGMP and 2 mM $MnCl_2$, 10 μg of creatine kinase, 10 mM creatine phosphate and 0.4 mM [8-^{3}H] GTP (0.02 μCi/nmol) in a total volume of 0.15 ml.

This mixture was incubated for 10 min at 37°C. The incubation started with the addition of [^{3}H]GTP and terminated by heating for 2 min in a boiling bath. The cGMP formation with this method was linear for 20 min.

2.3. *Isolation of cyclic GMP*

The radioactive cyclic GMP which was formed by guanylate cyclase was isolated with column chromatography as described by Mao and Guidotti [18].

2.4. *Preincubation of cerebellar membrane preparations*

In order to measure the inhibitory effect of cAMP dependent protein kinase on guanylate cyclase activity, 200 μl of soluble or particulate membrane preparations were preincubated in the presence or absence of the protein kinase at 30°C for 2, 4 and 10 min before guanylate cyclase assay. The preincubation system contained 10 mM $(CH_3COO)_2Mg$ (pH 6.0), 10 mM CH_3COONa (pH 6.0), 1 μM ATP and/or 1 μM cAMP in a final volume of 450 μl. The reaction started with the addition of ATP and $(CH_3COO)_2Mg$. At the end of the preincubation period 450 μl of the same incubation medium used for guanylate cyclase assay were added, and the system was immediately chilled in ice. 100 μl of the mixture thus obtained were then employed for measurement of guanylate cyclase activity.

2.5. *Protein*

Protein was measured according to Lowry et al. [19] using bovine serum albumin as a standard.

3. Results

As shown in fig.1, preincubation of the enzyme preparation with ATP or cAMP separately, or ATP and cAMP in the absence of protein kinase did not induce a relevant inactivation of guanylate cyclase compared to the activity measured in the standard assay system. Moreover, after 2 min of preincubation in the presence of protein kinase the enzyme activity

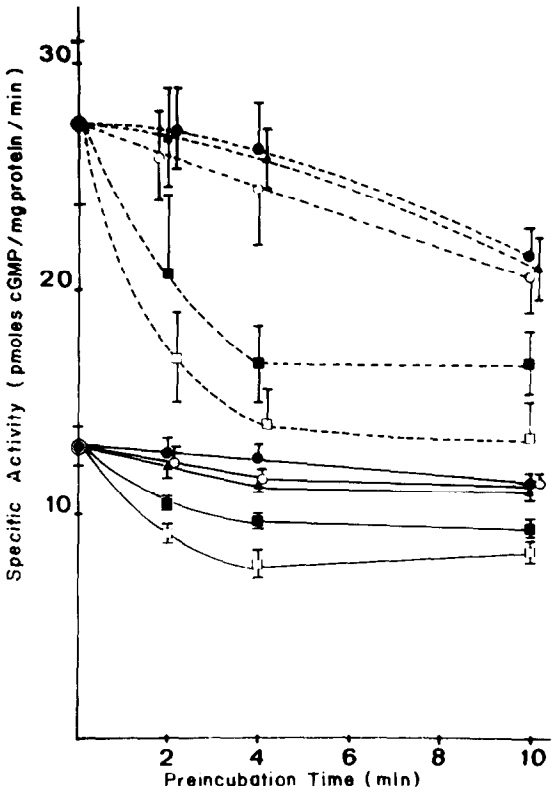

Fig.1. Guanylate cyclase activity after preincubation with or without protein kinase in the phosphorylation system. Guanylate cyclase in supernatant (——) and particulate (- - - -) fractions of rat cerebellar homogenates was preincubated at 30°C for various periods in the presence of: 1 μM ATP (●); 1 μM cyclic AMP (▲); 1 μM ATP and 1 μM cyclic AMP (○); 1 μM ATP, 1 μM cyclic AMP and 35 μg of protein kinase (■); 1 μM ATP, 1 μM cyclic AMP and 70 μg of protein kinase (□). Enzyme activity was expressed as pmol of cyclic GMP formed/mg protein/min. Each value was calculated on the basis of ten enzyme determinations.

was markedly decreased both in soluble and particulate fractions. The maximal inactivation by about 40% occurred after 4 min of preincubation and lasted for at least 10 min. On the other hand, this inactivation was further increased by the addition to the preincubation mixture of a larger amount of protein kinase (70 μg). Furthermore protein kinase denatured by heating in boiling water for 4 min was not able to produce any significant inactivation of guanylate cyclase, even if preincubation was performed in the presence of 10^{-6} M cAMP.

Figure 2 shows the effect of various cAMP concentrations on the inactivation elicited by protein kinase on guanylate cyclase activity. Particulate guanylate cyclase showed a clear cAMP dependent inactivation for cAMP concentrations ranging from 10^{-8} M to 10^{-6} M. The effect of different cAMP concentrations on the inactivation of the soluble enzyme was also observed. On the other hand, preincubation with protein kinase without cAMP caused by itself an inactivation of 25% in the soluble fraction and of 20% in the particulate fraction.

In another experiment the effect of NaN_3 on guanylate cyclase activity was studied and the results are shown in table 1. All the enzyme preparations were preincubated twice before guanylate cyclase assay. This procedure in the absence of both NaN_3 and protein kinase produced a slight decrease of the enzyme activity, about 10–15% compared to the activity measured with the standard assay system.

NaN_3 stimulated three fold the activity of the particulate enzyme and this effect was not blocked by the preincubation with protein kinase as demonstrated in table 1 (C and D). Moreover, when guanylate cyclase was first preincubated with protein kinase and then treated with 1 mM NaN_3 the activity of the particulate enzyme (E) was still three times higher than control activity. On the other hand NaN_3

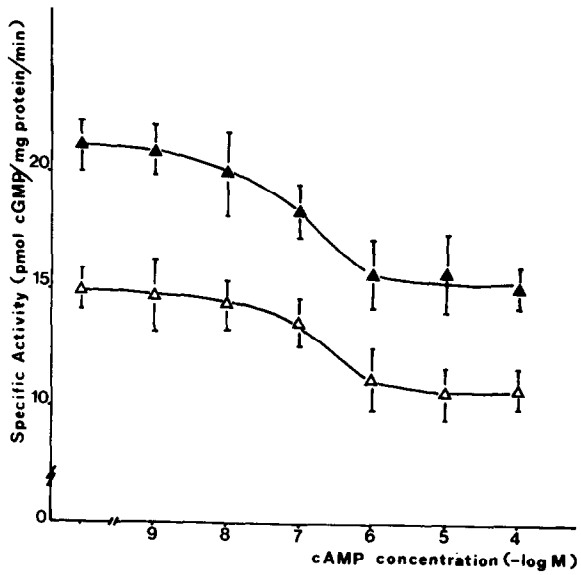

Fig.2. Guanylate cyclase activity after preincubation with protein kinase in the presence of cyclic AMP in various concentrations. Guanylate cyclase in soluble (△----△) and particulate (▲----▲) fraction of rat cerebellar homogenates was preincubated at 30°C for 4 min with 35 μg of protein kinase, 1 μM ATP, and cyclic AMP indicated in the phosphorylation system described in section 2. Enzyme activity was expressed as pmol of cyclic GMP (cGMP) formed/mg protein/min. Each value was calculated on the basis of eight enzyme determinations.

Table 1
Effect of preincubation with NaN_3 and protein kinase on guanylate cyclase activity

Addition to preincubation					
First preincubation	(A) none	(B) none	(C) NaN_3	(D) NaN_3	(E) protein kinase
Second preincubation	none	protein kinase	none	protein kinase	NaN_3
Soluble	17.7 ± 1.4	12.7 ± 1.0	19.6 ± 1.5	14.6 ± 1.4	13.3 ± 1.2
Particulate	28.6 ± 2.6	20.2 ± 1.1	83.9 ± 2.9	94.6 ± 3.9	93.2 ± 7.8

Enzyme preparations (A), (B), (C) and (D) were at first preincubated in the absence or presence of 1 mM NaN_3 at 37°C for 5 min; then were incubated again in a phosphorylation system at 30°C for 4 min as described in the Methods section in the presence or absence of protein kinase. In the case of (E) enzyme preparation was preincubated in the phosphorylation system with protein kinase and then treated with 1 mM NaN_3. Guanylate cyclase activity is expressed as pmol of cGMP formed/min/mg protein. Each value is calculated on the basis of eight enzyme determinations

neither affected the basal activity, nor changed the inhibitory effect of the cAMP dependent protein kinase in soluble guanylate cyclase preparations.

4. Discussion

The results of our study demonstrate that a cAMP dependent protein kinase can inactivate guanylate cyclase both in 105 000 × *g* supernatant and particulate fractions of rat cerebellar homogenates. This inactivation is probably induced by the phosphorylating action of the protein kinase, since this inhibition is dependent upon cAMP concentrations. In fact it has been demonstrated that cAMP dependent protein kinase is maximally stimulated by 10^{-6} M–5×10^{-6} M cAMP [15,16,20].

Our results are in good agreement with these data, since the maximal inactivation of guanylate cyclase was obtained in the presence of 10^{-6} M cAMP. It is still unclear whether this inhibition is caused by phosphorylation of the guanylate cyclase system or by the interference with some protein inhibitory factors.

Our data showing that NaN_3, an activator of guanylate cyclase activity in several tissues [10,21–23], is able to block the inactivation of the particulate enzyme, may be an indication for the reversibility of the inhibitory effect of protein kinase. These data may suggest that a site for a phosphorylation, which seems to be responsible for the inactivation of the enzyme, could be closely related to a site, where NaN_3 affects guanylate cyclase activity. NaN_3 is a strong nucleophylic substance, and some other nucleophylic agents have been also reported to activate guanylate cyclase [10].

In conclusion our results suggest an involvement of a phosphorylating system in the regulation of cGMP levels in cerebellum. Other studies are in progress to determine if this system is operant also in other CNS areas, where guanylate cyclase activity has been detected and correlated with neuronal activities [24].

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