Cyclic AMP-dependent protein kinase does not phosphorylate cyclic GMP-dependent protein kinase in vitro

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The autophosphorylation reaction of purified cGMP-dependent protein kinase has been studied. Apparent initial rates of autophosphorylation in the absence of cyclic nucleotides and in the presence of cGMP and cAMP are 0.006, 0.04, 0.4 mol P_i incorp./min^{-1}.mol cGMP-kinase subunit^{-1}. In the presence of cGMP and cAMP ~1 and 2 mol P_i are incorporated/mol enzyme subunit. These values are independent of the enzyme concentration. Stimulation of autophosphorylation by cAMP is not due to activation of a contaminating cAMP-dependent protein kinase since: (a) addition of the heatstable inhibitor protein of cAMP-kinase does not inhibit autophosphorylation; and (b) catalytic subunit of cAMP-kinase added at a 10-fold excess over cGMP-kinase does not phosphorylate cGMP-kinase.

cGMP-dependent protein kinase *cAMP-dependent protein kinase* *Autophosphorylation* *Heat-stable inhibitor protein*

1. INTRODUCTION

Cyclic GMP-dependent protein kinase (cGMP-kinase) is a dimer of presumably identical subunits (M_r 75000–81000) [1,2] and shares several properties with type II cAMP-dependent protein kinase (cAMP-kinase) including a similar M_r, amino acid composition, number of binding sites (4/mol holoenzyme) [3] and the ability to phosphorylate itself [4]. Autophosporylation of the pure cGMP-kinase has been reported to be stimulated slightly by cGMP [3,5], inhibited by cGMP [4,6,7] and free regulatory subunit of type I cAMP-kinase [8], to be stimulated by cAMP [4–9] or to proceed in the absence of added cyclic nucleotides [6]. Recently it has been shown that in the presence of cGMP and cAMP slightly <1 mol, and slightly >2 mol phosphate are incorporated/mol subunit, respectively [5,9]. Here, we show that the initial rate of autophosphorylation is slow in the presence of cGMP, and rapid in the presence of cAMP. This marked difference of cAMP and cGMP to alter the rate and the stoichiometry of the autophosphorylation process and the ability of regulatory subunit I to inhibit the autophosphorylation only in the absence of cAMP, raised the possibility that cAMP affected the phosphorylation of cGMP-kinase, not by binding to and activation of the cGMP-kinase itself, but by activation of a contaminating cAMP-kinase. Experiments carried out in vitro in the absence and presence of the catalytic subunit of cAMP-kinase and the heat-stable inhibitor protein of cAMP-kinase (PKI) [10] show that cGMP-kinase is not phosporylated by cAMP-kinase, even if the catalytic subunit of cAMP-kinase is added at a 10-times higher concentration than cGMP-kinase. This suggests that cAMP stimulates phosphorylation of cGMP-kinase not by activation of a contaminating cAMP-kinase but by activation of cGMP-kinase itself.

2. METHODS

Cyclic GMP-kinase was purified from bovine lung as in [9], using 5 mM cAMP at 22°C for elution of the enzyme from the cGMP–Sepharose [11]. Bound cAMP was removed from the enzyme by gel filtration at 22°C as in [9]. Catalytic subunit

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of type II cAMP-kinase was purified from bovine heart muscle [12]. Purity of the enzyme was >95% as determined from the Coomassie blue stains of SDS–polyacrylamide gels. PKI was purified from rabbit skeletal muscle as in [10] including the Sephadex G-100 step. The protein kinase activity was determined as in [13] using the substrate peptide as phosphate acceptor. Autophosphorylation reactions were carried out at 30°C in a 50 mM Mes buffer (pH 7.0) containing 0.37 mM EGTA, 180 mM NaCl, 0.13 mg/ml bovine serum albumin, 12 mM magnesium acetate, 0.12 mM [γ-^{32}P]ATP and enzymes as indicated. When present, cGMP and cAMP are 12 and 120 μM, respectively. Autophosphorylation reactions were stopped by the addition of trichloroacetic acid as in [9]. All values shown are based on a cGMP-kinase subunit M_r of 75000. For further methods and materials see [9].

3. RESULTS AND DISCUSSION

It has been shown [9] that the autophosphorylation of cGMP-kinase is stimulated slightly by cGMP and more strongly by cAMP. As shown in table 1, cGMP and cAMP increased the initial phosphorylation rate of pure cGMP-kinase about 5- and 50-times, respectively. The absolute values of these rates varied about 3-fold between different enzyme preparations, but the phosphorylation rate was always stimulated 10-times as much by cAMP than by cGMP. The initial rates, the relative potency of cGMP and cAMP to stimulate autophosphorylation and the amount of phosphate incorporated in the presence of cGMP or cAMP were independent of the enzyme concentration (table 1). The phosphorylation stoichiometry did not change significantly if the incubation time was prolonged to 3 h indicating that they reflected the maximal number of sites available for phosphorylation in the presence of cGMP and cAMP. These marked differences in the initial rates and stoichiometry elicited by cGMP and cAMP can be explained if it is assumed that both cyclic nucleotides bind to the same sites, and activate in principle the same catalytic site on cGMP-kinase, but that they induce different conformational states of the enzyme which are responsible for the observed differences in the initial rates and stoichiometry. An alternative and more simplistic explanation would be that cAMP binds to, and activates a cAMP-kinase which contaminates the cGMP-kinase preparation and phosphorylates cGMP-kinase more efficiently than cGMP-kinase phosphorylates itself in the presence of cGMP.

This latter possibility was tested by adding low concentrations of free catalytic subunit of cAMP-kinase to cGMP-kinase in the absence and presence of cGMP and cAMP (fig.1). No effect of the cata-

Table 1

Initial rates and stoichiometry of autophosphorylation of cGMP-kinase

Subunit conc. (pmol)	Initial rate			Stoichiometry		
	+ H_2O	+ cGMP	+ cAMP	+ H_2O	+ cGMP	+ cAMP
	(mol phosphate . mol subunit^{-1} . min^{-1})			(mol phosphate . mol subunit^{-1})		
Expt A						
10.0	0.006	0.04	0.4	0.4	0.7	2.4
Expt B						
13.9	–	0.020	0.25	–	1.2	2.2
46.4	–	0.018	0.20	–	1.1	1.8
139.0	–	0.019	0.20	–	1.0	2.2
464.0	–	0.020	0.24	–	0.8	1.8

The initial rates were calculated from 3 time points taken for 0–3 min (expt A) and 0–5 min (expt B). The stoichiometry of phosphate incorporation was determined after 50 min (expt A), and 40 min (expt B). Two different enzyme preparations were used for expt A and B. Total incubation vol. was 100 μl

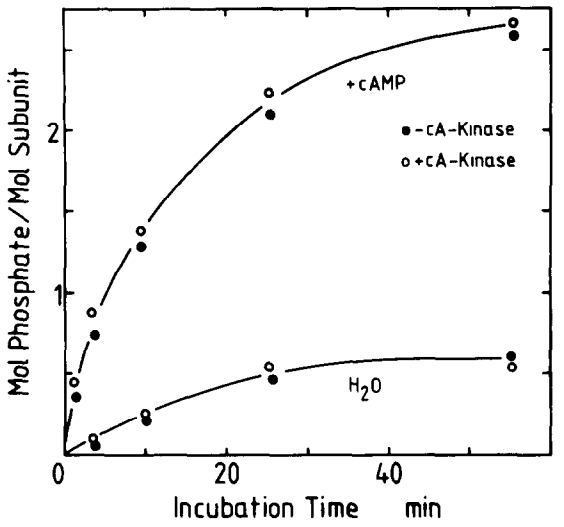

Fig.1. Cyclic GMP-kinase (11 pmol) was incubated in the absence and presence of cAMP. Identical tubes were incubated in the absence and presence of catalytic subunit of cAMP-kinase (10 pmol). Total incubation vol. was 100 μl. Values obtained in the absence of cGMP-kinase have been subtracted.

Table 2

Phosphorylation of cGMP-kinase is not affected by the heat-stable inhibitor protein of cAMP-kinase (PKI)

Nucleotide	cAMP-kinase	− PKI (mol phosphate. mol subunit)	+ PKI (mol phosphate. mol subunit)
None	−	0.42	0.41
	+	0.46	0.41
cGMP	−	0.53	0.57
	+	0.42	0.55
cAMP	1	2.68	2.53
	+	2.61	2.62

Phosphorylation of cGMP-kinase (11 pmol) was determined in the absence and presence of cGMP or cAMP. To some tubes, 3.3 μl PKI, or 5 pmol catalytic subunit of cAMP-kinase were added alone or together. This amount of PKI completely inhibited the activity of 20 pmol catalytic subunit of cAMP-kinase using the substrate peptide as phosphate acceptor. Total incubation vol. was 90 μl. Incubation time was 25 min. Values obtained in the absence of cGMP-kinase have been subtracted

lytic subunit of cAMP-kinase on the time course of the autophosphorylation reaction of cGMP-kinase in the absence of cyclic nucleotides or in the presence of cAMP or cGMP (see table 2) was observed. The failure to observe phosphorylation of cGMP-kinase by cAMP-kinase was not due to the low amount of catalytic subunit added. Variation of the concentration of catalytic subunit of cAMP-kinase, >100-fold, in the presence of a constant concentration of cGMP-kinase did not affect the amount of phosphate incorporated in the presence of cGMP or cAMP (fig.2). A slight enhancement of the phosphorylation observed in the absence of added cyclic nucleotides was apparent at the higher concentrations of cAMP-kinase. This experiment suggests that cAMP-kinase is not able to phosphorylate cGMP-kinase even if present in quantities 10-times as big as those of cGMP-kinase. The same result will be obtained when the catalytic subunit of cAMP-kinase is rapidly inactivated by cGMP-kinase or some constituents of the cGMP-kinase preparations. This possibility was tested by incubating both protein kinases alone or together in the absence and presence of the heat-stable inhibitor protein (PKI). The catalytic activity of each

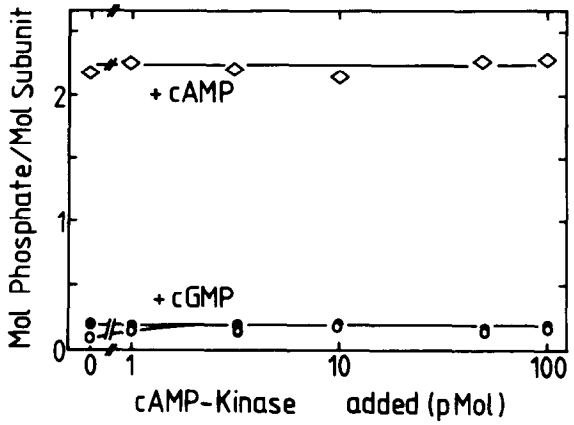

Fig. 2. cGMP-kinase (11 pmol) was incubated in the absence (○) and presence (●) of cGMP, and cAMP (◇). Catalytic subunit of cAMP-kinase was present at the identical concentration. Values obtained in the absence of cGMP-kinase have been subtracted. Total incubation vol. was 80 μl. Samples were incubated for 20 min.

enzyme was followed by the phosphorylation of the substrate peptide [13]. As expected, both enzymes phosphorylated the substrate peptide in

the absence of PKI. Addition of PKI inhibited the activity of cAMP-kinase and had no effect on the activity of the cGMP-kinase. Incubation of both enzymes, in the absence and presence of PKI, resulted in the appropriate activity values; e.g., in the absence of PKI the sum of the activities of both enzymes was obtained, and in the presence of PKI an activity corresponding to those values obtained with cGMP-kinase alone was observed. This pattern was not changed by addition of cAMP or cGMP, suggesting that the catalytic subunit of cAMP-kinase retained its catalytic activity in the presence of cGMP-kinase.

These data support the above results that added catalytic subunit of cAMP-kinase does not phosphorylate cGMP-kinase despite the fact that cAMP stimulates the apparent autophosphorylation reaction much more efficiently than cGMP. However, these data do not completely rule out the possibility that in the presence of cAMP, cGMP-kinase is phosphorylated by an endogenous cAMP-kinase which contaminates the cGMP-kinase preparation. This consideration would demand that we assume:

(i) That the sites phosphorylated by cAMP-kinase are only available if cGMP-kinase binds cAMP, since catalytic subunit of cAMP-kinase did not phosphorylate cGMP-kinase in the absence or presence of cGMP;
(ii) That the amount of endogenous cAMP-kinase is high enough to catalyze a maximal phosphorylation rate which can not be further enhanced by addition of catalytic subunit.

The hypothetical activation of a contaminating cAMP-kinase during cAMP-stimulated autophosphorylation has been tested by incubation of cGMP-kinase in the presence and absence of PKI. This protein inhibits the catalytic activity of cAMP-kinase but not that of cGMP-kinase. No effect of PKI on the amount of phosphate incorporated into cGMP-kinase was found (table 2). Once again, addition of catalytic subunit of cAMP-kinase in the absence and presence of PKI did not influence the amount of phosphate incorporated into cGMP-kinase. Further experiments showed that PKI did not change the time course of the autophosphorylation reaction determined in the presence of cGMP or cAMP.

Together these results indicate that cAMP-kinase does not participate in the phosphorylation reaction of cGMP-kinase in vitro. This result is surprising since many proteins unrelated to cAMP-regulated cellular functions will serve in vitro as substrate for cAMP-kinase [14]. It is not known why the cAMP stimulates the phosphorylation of cGMP-kinase better than cAMP does. It is possible that cAMP and cGMP induce different conformations of the enzyme which facilitate the expression of distinct phosphorylation states of the enzyme, or which facilitate the ability of an unknown protein kinase to phosphorylate cGMP-kinase. The physiological significance of this reaction is not evident at present, but its potential importance for cGMP-regulated functions is supported by the observation that phosphorylation of cGMP-kinase is a rapid process, and that phosphorylation is accompanied by a 10-fold increase in the apparent affinity of the enzyme towards cAMP [9]. This change in affinity could be responsible for the recent observation that in tracheal smooth muscle a cGMP-dependent protein kinase activity is stimulated by μM concentrations of cAMP [15].

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