

Inhibition of cGMP-dependent protein kinase by (Rp)-guanosine 3',5'-monophosphorothioates

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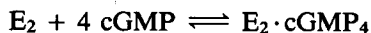
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The activation of the cGMP-dependent protein kinase and cAMP-dependent protein kinase by the diastereomers of guanosine 3',5'-monophosphorothioate, (Sp)-cGMPS and (Rp)-cGMPS, and 8-chloroguanosine 3',5'-monophosphorothioate, (Sp)-8-Cl-cGMPS and (Rp)-8-Cl-cGMPS, was investigated using the peptide Kemptide as substrate. The (Sp)-diastereomers, which have an axial exocyclic sulfur atom, bound to the cGMP-dependent protein kinase and stimulated its phosphotransferase activity. In contrast, the (Rp)-isomers, which have an equatorial exocyclic sulfur atom, bound to the enzyme without stimulation of its activity. (Rp)-cGMPS and (Rp)-8-Cl-cGMPS antagonized the activation of the cGMP-dependent protein kinase with a K_i of 20 μ M and 1.5 μ M, respectively. (Rp)-cGMPS also antagonized the activation of cAMP-dependent protein kinase with a K_i of 20 μ M. In contrast, (Rp)-8-Cl-cGMPS was a weak inhibitor of the cAMP-dependent protein kinase with a K_i of 100 μ M. (Rp)-8-Cl-cGMPS appears to be a rather selective inhibitor of the cGMP-dependent protein kinase and may be a useful tool for studying the role of cGMP in broken and intact cell systems.

Protein kinase, cGMP-dependent; Antagonist; cGMPS; 8-Cl-cGMPS

1. INTRODUCTION

cGMP-dependent protein kinase (cGK) from bovine lung is a dimer composed of two identical subunits of 75 kDa each [1]. cGK is considered to be important for the regulation of cerebellar Purkinje cells, smooth muscle cells, platelets and intestinal epithelial cells [2]. Activation of this enzyme occurs upon binding of four molecules of cGMP according to the following equation [3]:



Each cGK monomer contains one catalytic site and two cGMP-binding sites with different affinities for cGMP and its analogs [4]. In our search for useful inhibitors for cGK we synthesized (Rp)- and (Sp)-cGMPS as well as (Rp)- and (Sp)-8-Cl-cGMPS using a new method [5] which improved the yield of these nucleotides in comparison to that obtained by other methods reported earlier [6–8]. Additional reactions

were used to remove trace contaminants of cGMP. The selection of the stereoselective thiomodification was based on our experience with the homologous cAMP-dependent protein kinase (cAK). (Rp)-cAMPS is an antagonist of cAK and thus of cAMP-regulated processes including cAMP-regulated gene expression [9–13]. Recently, interaction of phosphorothioate derivatives of cGMP with the phosphodiesterase and the light-sensitive channel from rod outer segments [14] as well as the effects of these analogs on starfish oocyte maturation [12] were reported. In this study, the effects of highly purified diastereomers of cGMPS and 8-Cl-cGMPS on the activity of cGK and type II cAK were investigated.

2. MATERIALS AND METHODS

2.1. Materials

The cGK and type II cAK (EC 2.7.1.37) were purified from bovine lung and bovine heart, respectively, as described previously [15]. All cyclic nucleotides were synthesized and purified in our laboratory [5,16]. The purity was >99.8% as observed by HPLC, and all phosphorothioates were free of contamination by the corresponding cyclic nucleotides within the detection limit of HPLC. [γ - 32 P]ATP was from Amersham and the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) was purchased from Peninsula. Calmodulin-sensitive cyclic nucleotide phosphodiesterase (cPDE) from bovine heart (EC 3.1.4.1) was obtained from Sigma Chemicals. Centricon 10 was from Amicon GmbH, Witten, FRG.

2.2. cPDE assay

(Rp)-cGMPS and (Rp)-8-Cl-cGMPS are resistant to hydrolysis by different cPDEs [14,17,18]. Therefore, cGMP impurities can be

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Abbreviations: cAK, cAMP-dependent protein kinase; cGK, cGMP-dependent protein kinase; 8-Cl-cGMP, 8-chloro-8-cGMP; (Rp)- and (Sp)-cGMPS, phosphorothioate stereoisomers of cGMP; (Rp)- and (Sp)-8-Cl-cGMPS, phosphorothioate stereoisomers of 8-chloro-cGMP; (Rp)- and (Sp)-cAMPS, phosphorothioate stereoisomers of cAMP; cPDE, calmodulin-sensitive cyclic nucleotide phosphodiesterase

removed by incubation with this enzyme [19]. For complete elimination of traces of cGMP in the cGMP derivatives, 1 μ mol of the analog was incubated in the presence of 1 U/ml cPDE in 50 mM Tris/HCl buffer (pH 7.5), 0.1 mM $MgCl_2$ for 30 min at 30°C. At the end of the incubation, the nucleotide was separated from the enzyme by filtration using Centricon 10. This treatment significantly reduced the partial activation of cGK which was variably observed with high concentrations (50 μ M and higher) of some (Rp)-cGMP analogs tested.

2.3. Protein kinase assay

Protein kinase activity of cGK or cAK was measured using the phosphocellulose method [20] with minor modifications. Briefly, protein kinase activity was assayed at 30°C in a total volume of 100 μ l containing 20 mM Tris/HCl buffer (pH 7.4), 10 mM $MgCl_2$, 5 mM β -mercaptoethanol, 0.01% (w/v) bovine serum albumin, 10 μ g Kemptide (130 μ M), 50 or 100 ng protein kinase and cyclic nucleotides as indicated. The reaction was started by the addition of 50 μ M [γ - ^{32}P]ATP (about 100 cpm/pmol) and terminated after 5 min by the addition of 0.1 M EDTA. Activity is expressed as amount of phosphate transferred per min and mg protein kinase.

3. RESULTS AND DISCUSSION

The structures of the cGMP-analogs are shown in Fig. 1. The concentration-dependent effects of cGMP, (Rp)-cGMPS and (Sp)-cGMPS on the activity of cGK are demonstrated in Fig. 2. The apparent activation constants K_a (cyclic nucleotide concentration required for half-maximal activation) as well as the relative activation constants K'_a [$K'_a = K_a(\text{cGMP})/K_a(\text{analog})$] for the cyclic nucleotides tested are listed in Table I. The relative activation constant describes the activation potency of an analog compared to cGMP as activator. The data indicate that (Sp)-cGMPS is an agonist with respect to the activation of cGK although it is 135 times less potent than cGMP (Table I). In contrast, (Rp)-cGMPS does not activate the cGK (Fig. 2) and is a competitive inhibitor ($K_i = 20 \mu$ M) for the cyclic nucleotide-dependent activation of cGK (Fig. 3). Therefore, (Rp)-cGMPS binds to the cGK, but apparently does not cause the conformational change of the enzyme required for its activation. Similar effects

Table I

Activation and inhibition constants for bovine lung cGK

Cyclic nucleotide	Bovine lung cGMP-dependent protein kinase		
	K_a (M)	K'_a	K_i (M)
cGMP	2.0×10^{-7}	1.0	
(Sp)-cGMPS	2.7×10^{-5}	0.007	
(Rp)-cGMPS	antagonist	—	2×10^{-5}
8-Cl-cGMP	7.3×10^{-8}	2.7	
(Sp)-8-Cl-cGMPS	3.4×10^{-6}	0.057	
(Rp)-8-Cl-cGMPS	antagonist	—	1.5×10^{-6}
cAMP	3.9×10^{-5}	0.005	
(Sp)-cAMPS	antagonist	—	
	(partial agonist)	—	7.3×10^{-5}
(Rp)-cAMPS	antagonist	—	5.3×10^{-5}

Apparent activation constants K_a (concentrations required for half-maximal activation), relative activation constants K'_a [$K'_a = K_a(\text{cGMP})/K_a(\text{analog})$], or inhibition constants K_i of cGMP and some analogs for bovine lung cGK. Similar results were obtained in three separate experiments. The data for (Sp)-cAMPS and (Rp)-cAMPS were reported by Hofmann et al. [21]

were observed when 8-Cl-substituted derivatives of cGMPS were used. (Sp)-8-Cl-cGMPS activated the cGK with a K_a of 3.4 μ M while the (Rp)-diastereomer was an antagonist with a K_i of 1.5 μ M (Table I). As observed with cGMP, 8-Cl-substitution of (Sp)-cGMPS increased the affinity of this analog for the cGK (Table I); [17]).

The (Sp)-diastereomers which have an axial exocyclic sulfur atom in the cyclic phosphate moiety bound to the cGK with a reduced affinity and acted as agonists. (Rp)-derivatives with an equatorial exocyclic sulfur atom bound to the enzyme and were antagonists. The same effect has previously been observed with the cAK I and cAK II and the (Sp)- and (Rp)-diastereomers of cAMPS. Interestingly, both (Sp)- and (Rp)-phosphorothioate stereoisomers of cAMP are known to antagonize the activation of cGK [21], although with rather low inhibition constants when compared to

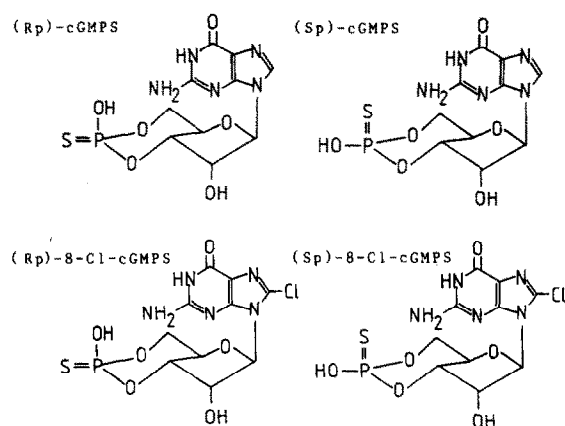


Fig. 1. Structures of (Rp)- and (Sp)-3',5'-monophosphorothioates of cGMP and 8-Cl-cGMP.

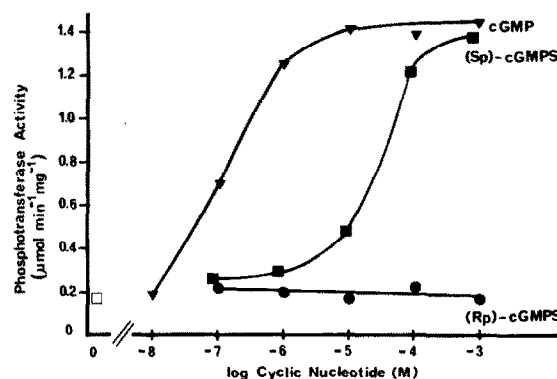


Fig. 2. Effects of cGMP (▼), (Sp)-cGMPS (■), and (Rp)-cGMPS (●) on the activity of cGMP-dependent protein kinase. The activity of the enzyme in the absence of cyclic nucleotides is indicated (□). Similar data were obtained in several separate experiments.

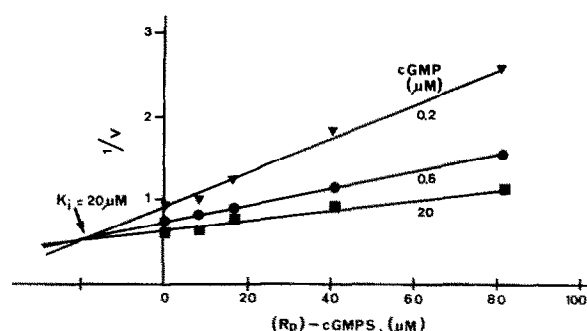


Fig. 3. Dixon plot demonstrating the competitive inhibition by (Rp)-cGMPS of the cGMP-induced activation of cGK. $1/V$ is the reciprocal value of the phosphotransferase activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) measured. Similar results were obtained in three separate experiments.

(Rp)-8-Cl-cGMPS (Table I). These data indicate that the exocyclic oxygens of the cyclic phosphate are very important for the mechanism of binding and activation of cyclic nucleotide-dependent protein kinases. In this respect, cAK and cGK have similar properties. Therefore, the phosphorothioate stereoisomers of cGMP were also tested with respect to their effects on the type II cAK. Surprisingly, (Sp)-stereoisomers of cGMPS and 8-Cl-cGMPS were better activators of cAK than cGMP and 8-Cl-cGMP, respectively (Table II). (Rp)-cGMPS and (Rp)-8-Cl-cGMPS were inhibitors of bovine heart type II cAK, however, they had a reduced binding affinity when compared to (Rp)-cAMPS (Table II).

In conclusion, (Rp)-cGMPS is a moderately effective antagonist for both cGK and cAK while (Rp)-8-Cl-cGMPS is a potent and reasonably selective inhibitor of the cGK. (Rp)- and (Sp)-cGMPS as well as the 8-Cl-

substituted diastereomers of these nucleotides should be valuable tools for the investigation of cGMP-regulated biological processes. In particular, this will be of interest with respect to cGMP-regulated cyclic nucleotide phosphodiesterases [23] and cGMP-regulated ion channels [24] as well as other types of cGMP-dependent protein kinase [2] than the one studied here. Phosphorothioate analogs such as (Sp)- and (Rp)-8-Cl-cGMPS are not only effective activators or inhibitors, respectively, of cGK (this study), but these analogs are also resistant to hydrolysis by phosphodiesterases and have a lipophilicity which is similar to that of 8-bromo-cGMP and much higher than that of cGMP [17]. Therefore, these diastereomers should be of considerable interest for studies investigating cGK-controlled functions with intact cells such as regulation of smooth muscle calcium concentration or nitrovasodilator-induced protein phosphorylation [25,26].

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Table II

Activation and inhibition constants for bovine heart type II cAK

Cyclic nucleotide	Type II cAMP-dependent protein kinase		
	K_a (M)	K'_a	K_i (M)
cAMP	1×10^{-7}	1.0	
cGMP	6×10^{-5}	0.0016	
(Sp)-cGMPS	2.7×10^{-5}	0.003	
(Rp)-cGMPS	antagonist	—	2×10^{-5}
8-Cl-cGMP	6.5×10^{-5}	0.0015	
(Sp)-8-Cl-cGMPS	8.3×10^{-6}	0.012	
(Rp)-8-Cl-cGMPS	antagonist	—	10×10^{-5}
(Sp)-cAMPS	1.8×10^{-6}	0.056	
(Rp)-cAMPS	antagonist	—	3.7×10^{-6}

Apparent activation constants K_a (concentrations required for half-maximal activation), relative activation constants K'_a [$K_a(\text{cAMP})/K_a(\text{analog})$], or inhibition constants K_i of cAMP, cGMP and some analogs for bovine heart type II cAK. Similar results were obtained in three separate experiments. The data for (Sp)-cAMPS and (Rp)-cAMPS were reported by Van Haastert et al. [22] and were obtained with type II cAK from rabbit muscle

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