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A stimulating effect of guanyl nucleotides on the rat-liver soluble cyclic GMP high-affinity phosphodiesterase activity

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The high affinity (low K_m) cyclic GMP phosphodiesterase (PDE) is activated by GTP, while the cyclic AMP PDE is not. GTP and its hydrolysis-resistant analogue, guanylylimidodiphosphate (GppNHp), display a half-maximal stimulating effect at almost the same concentration (5×10^{-6} M). The GTP stimulating effect is not observed when the socalled cyclic GMP low affinity (high K_m) PDE is operative. GTP cooperates with the increase of the substrate concentration on removing the IBMX inhibitory effect. The isolation through a classical chromatographic procedure on a DEAE-cellulose column, of a PDE fraction specific for cyclic GMP, results in the loss of the GTP stimulating effect.

Soluble phosphodiesterase *Cyclic nucleotide* *Guanosine nucleotide* *(Liver)*

1. INTRODUCTION

Cyclic AMP and cyclic GMP appear to mediate many alternative cellular processes (e.g., differentiation and proliferation), while their cellular levels appear to be regulated in a reciprocal and alternative way, so that to an increase of cyclic AMP corresponds a decrease in the cyclic GMP content and vice versa [1,2].

The occasional finding of a stimulating effect of GTP on the rat liver soluble cyclic GMP phosphodiesterase (PDE) activity urged us to explore the effects of guanyl nucleotides on cyclic GMP catabolism, foreseeing the possibility of an efficient control mechanism, based on a common positive effector, GTP, for cyclic AMP biosynthesis and for cyclic GMP breakdown.

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Abbreviations: PDE, cyclic nucleotide phosphodiesterase (EC 3.1.4.17); HPLC, high-pressure liquid chromatography; GppNHp, 5′-guanylylimidodiphosphate; Gpp(CH_2)p, β,γ methylene-guanosine-5′-triphosphate; IBMX, 3-isobutyl-1-methylxanthine

To date, a stimulating GTP effect has been shown only on a retinal rod outer-membrane-bound cyclic GMP phosphodiesterase, described as linked to a GTPase activity [3,4].

2. MATERIALS AND METHODS

Male Sprague-Dawley rats (150–200 g body wt) were killed by decapitation; livers were removed and perfused through the portal vein with 30 ml ice-cold 50 mM Tris–HCl buffer (pH 8.0) containing 0.25 M sucrose. Subsequent procedures were performed at 4°C. The livers were homogenized for 30 s in two successive strokes, in 4 vol. of the above buffer, in a Potter Teflon–glass apparatus. The homogenate was centrifuged at $800 \times g$ for 15 min. The sediment was discarded and the resulting supernatant was recentrifuged at 105000 $\times g$ for 1 h. The supernatant was used as enzymic source for PDE activity.

For the determination of PDE activity a two-step radioassay was used [5]. In the first step, the incubation mixture contained, in 100 μl final vol.: 4 mM $MgCl_2$, 2 mM 2-mercaptoethanol, cyclic [^{3}H]GMP or cyclic [^{3}H]AMP (spec. act.

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24 Ci/mmol; about 60000 cpm), 25 mM Tris–HCl buffer (pH 7.5) and various concentrations of cyclic GMP or cyclic AMP. The reaction was started by the addition of the enzymic source (80–100 μg) and after a 15 min incubation at 37°C was stopped by boiling for 3 min. After cooling, 25 μl *Ophiophagus hannah* venom (1 mg/ml) was added to the reaction mixture and incubation was carried out for 10 min at 37°C to achieve complete conversion of the produced linear nucleotide either to guanosine or adenosine, according to the substrate used (cyclic GMP or cyclic AMP). After this step, 400 μl AG1X2 resin (200–400 mesh, Cl^- form) as resin/water (1 : 1) was added up, and after extensive vortexing the samples were centrifuged in a Beckman Microfuge at 12000 × *g* for 15 min. Samples (100 μl) of the supernatant were removed for determination of the radioactive guanosine or adenosine present in the solution.

To determine the interference of the substances studied on the nucleotidase step and on the binding of [^{3}H]guanosine and [^{3}H]adenosine by the resin, control assays were performed by adding nucleotides and nucleosides after the first incubation. Corrections were also made for the cyclic [^{3}H]GMP or cyclic [^{3}H]AMP not removed by the resin, by using a boiled enzyme preparation as a blank. Moreover, we also tested the effects of the compounds studied on the PDE by isolation both of the reactants and the products of the reaction, through HPLC (Beckman Altex apparatus equipped with a reverse phase ODS column, 0.46 × 25 cm, equilibrated with 20 mM KH_2PO_4, containing 1% methanol).

The purity of the compounds used was checked by HPLC using the procedure indicated above, and corrections were made for the presence of contaminating nucleosides and nucleotides.

3. RESULTS

The results reported in table 1 show that the so-called cyclic GMP high affinity PDE activity of rat liver supernatant can be stimulated by guanyl nucleotides and nucleosides, while the so-called cyclic AMP high affinity phosphodiesterase activity is not stimulated. While the hydrolysis of cyclic GMP at 10^{-4} M can be increased up to 65% above the control, no effect is observed whenever cyclic AMP is used as substrate. GTP appears to be more effective either than dGTP or the hydrolysis-resistant analogues GppNHp and Gpp(CH_2)p. Also GDP, GMP and guanosine appear to be effective, but to a different extent, on the enzymic activity investigated.

Table 1

The effects of guanyl nucleotides and nucleoside on the soluble cyclic GMP and cyclic AMP PDE activities

Addition (10^{-4} M)	PDE activity (%)	
	Cyclic GMP[a] (10^{-6} M)	Cyclic AMP[b] (10^{-6} M)
None	100	100
dGTP	141	103
GTP	165	96
Gpp(NH)p	151	98
Gpp(CH_2)p	165	92
GMP	135	115
Guanosine	116	105

The control activity: [a] in the presence of cyclic GMP was 18 pmol 5′-GMP formed.min^{-1}.mg $protein^{-1}$; [b] in the presence of cyclic AMP it was 25 pmol 5′-AMP formed.min^{-1}.mg $protein^{-1}$

Values are averages of triplicate determinations in two experiments, which agreed to within 5%

The incubation mixture for PDE activity contained (100 μl): 4 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 25 mM Tris–HCl buffer (pH 7.5), 10^{-6} M cyclic GMP or cyclic AMP, [^{3}H]cyclic GMP or cyclic AMP (~60000 cpm) and the compound tested at 10^{-4} M final conc. The reaction was started by the addition of the enzymic source (80–100 μg), continued for 15 min at 37°C and stopped by boiling for 3 min. The 5′-GMP (or 5′-AMP) formed was assayed according to Thompson and Appleman [5]. For other experimental conditions, see section 2

Controls were performed to rule out a guanyl nucleotide effect on the enzymic assay we used to convert 5′-GMP or 5′-AMP to guanosine or adenosine respectively, or on the chromatographic procedure.

Fig.1 indicates the correlation between the GTP (●—●) or GppNHp (▲—▲) concentrations and the stimulating effect on the PDE when 10^{-6} M cyclic GMP is used as substrate. It is shown that 10^{-4} M of both nucleotides allows the maximum stimulating effect to be obtained and that the

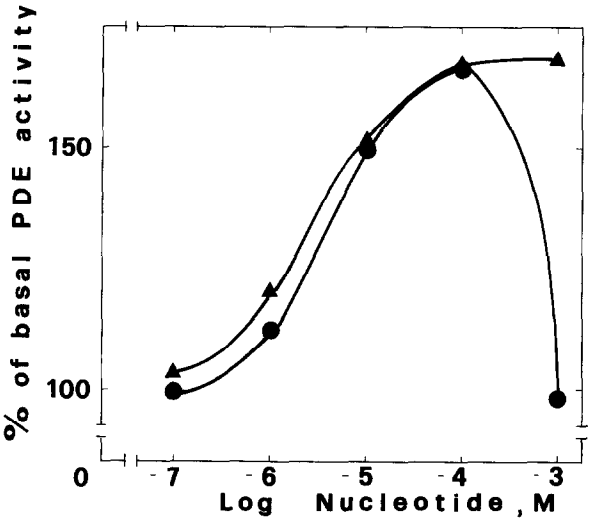

Fig.1. Dose–effect relationship of GTP and GppNHp on rat-liver soluble cyclic GMP high affinity PDE: (●—●) + GTP; (▲—▲) + GppNHp. Results are expressed as percent of the control (no addition) activity. Experimental points are averages of triplicates which differed by ⩽5%. For experimental conditions see section 2.

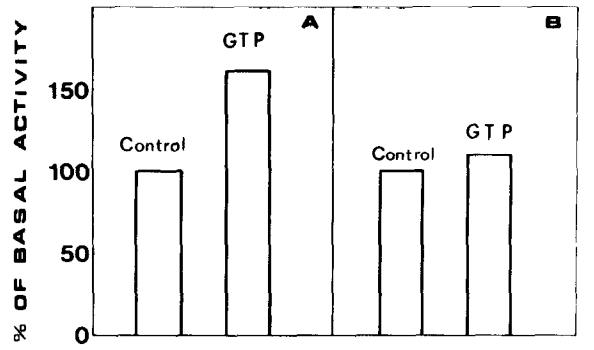

Fig.2. The effects of 10^{-4} M GTP on the rat-liver soluble cyclic GMP PDEs. The experiments were carried out in the presence of two cyclic GMP concentrations; for each cyclic GMP concentration the results are expressed as percentage of the control (no addition) activity: (A) 10^{-6} M cyclic GMP; the control activity corresponds to 18 pmol 5′-AMP.min^{-1}.mg protein^{-1}; (B) 10^{-3} M cyclic GMP; the control activity corresponds to 286 pmol 5′-GMP.min^{-1}.mg protein^{-1}. Results are the mean of 3 separate expt performed in triplicate, which differed by ⩽10%. For other experimental conditions see section 2.

hydrolysis-resistant analogue-stimulating effect is more regularly dependent on the concentration than the one due to GTP. The results in fig.2 indicate that the GTP-stimulating effect, while substantial in the presence of 10^{-6} M cyclic GMP, is almost irrelevant in the presence of 10^{-8} M cyclic GMP, the one that causes the so-called low affinity PDE activity to operate.

In fig.3 are reported the results of kinetic experiments carried out in the absence (○—○) or in the presence (□—□) of 10^{-4} M GTP. They indicate that GTP stimulates the enzymic activity investigated at every tested cyclic GMP concentration. Due to the presence of heterogeneous enzymic populations, we did not characterize the kinetic parameters (V_{max} and K_m).

Fig.4 consists of 6 panels reporting the effects of 10^{-4} M GTP on the inhibitory action of IBMX on PDE activity at 6 different cyclic GMP concentrations, ranging from 0.2×10^{-6} M (panel I) to 14×10^{-6} M (panel VI). The results reported show that the inhibitory effect of IBMX is removed not only by an increase of substrate concentration, but also by the presence of GTP. Moreover, in the presence of higher cyclic GMP concentrations (panels V,VI), which can, by themselves, remove the inhibitory effect of IBMX, IBMX even reinforces the GTP stimulation.

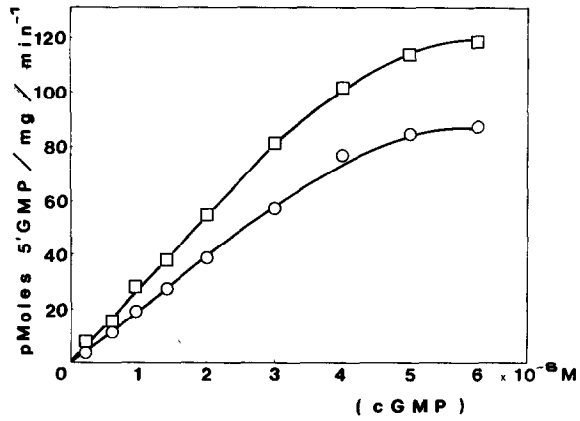

Fig.3. Kinetics of the rat-liver soluble cyclic GMP high affinity PDE. In our experimental conditions it is not possible to quantify the modifications of the kinetics parameters, due to the complicating interference introduced by higher substrate concentrations, when other populations of PDE become operative: (○—○) no addition; (□—□) + 10^{-4} M GTP. For experimental conditions see section 2.

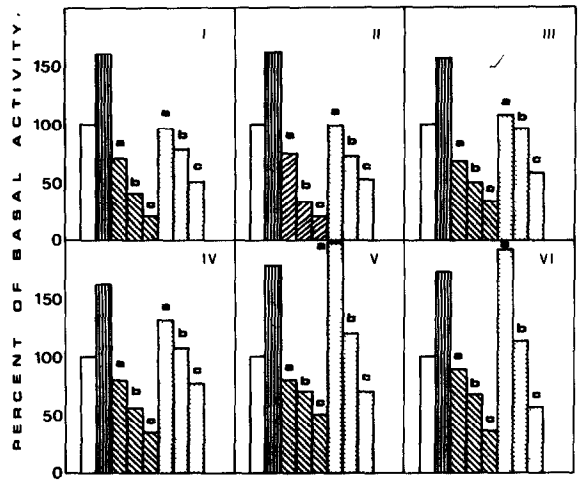

Fig.4. The effects of 10^{-4} M GTP on the basal and IBMX-inhibited rat-liver soluble cyclic GMP PDE: (□) basal activity; (■) + 10^{-4} M GTP; (▨) + IBMX (a, 0.1 mM; b, 0.5 mM; c, 1 mM); (▩) + IBMX (a, 0.1 mM; b, 0.5 mM; c, 1 mM) + 10^{-4} M GTP; (I) 0.2×10^{-6} M cyclic GMP; (II) 0.6×10^{-6} M cyclic GMP; (III) 1.4×10^{-6} M cyclic GMP; (IV) 2×10^{-6} M cyclic GMP; (V) 6×10^{-6} M cyclic GMP; (VI) 14×10^{-6} M cyclic GMP. The results indicate the means of two typical experiments, performed in triplicate, which differed by ≤5%. For other experimental conditions, see section 2.

The selectivity of the GTP stimulation on cyclic GMP high-affinity PDE activity, was checked on PDE, isolated by a described chromatographic separation on a DEAE–cellulose column [6]: unfortunately the 3 peaks of activity obtained were unresponsive to GTP (not shown).

4. DISCUSSION

The differential effects of the guanyl nucleotide on the hydrolyzing activities on cyclic nucleotides appear to have a regulatory relevance to the reciprocal control of the intracellular levels of cyclic AMP and cyclic GMP.

In fact, the stimulating effect of guanyl nucleotide on cyclic GMP hydrolysis appears to operate synergistically with the guanyl nucleotide effect on adenylate cyclase activity.

The major features of the stimulating effect of GTP on the cyclic GMP PDE activities are the following: it does not seem to be linked either to a high energy phosphate transfer or to a GTPase activity since it is shown by GTP, by its hydrolysis-resistant analogs and by the hydrolytic GTP derivatives (among them even guanosine is active). The presence of a GTPase activity could explain some results we obtained at the very low or very high concentrations of GTP we used. In fact, in fig.1, we observe that at 10^{-6} M GTP, a GTPase activity could explain the minor efficacy of GTP compared to GppNHp. Moreover, for the diminution of the stimulating effect obtained at very high GTP concentrations (10^{-3} M), either a GTP-hydrolyzing site with a lower affinity or a direct inhibitory effect on the catalytic site can be postulated. Eventually, we must be aware that a consistent guanylate cyclase activity is present in the soluble enzymic source used which, at the higher GTP concentrations, could hydrolyze enough unlabelled cyclic GMP to protect the labelled cyclic GMP we used as tracer substrate from hydrolysis.

Because of the efficacy of dGTP, the hydroxyl lacking in the ribose ring does not seem to be critical.

The stimulation appears to be devoted to the control of the fluctuations in μM cyclic GMP levels, the ones which usually occur in the cell and that trigger consistent metabolic shifts. In fact, in the presence of PDE already stimulated by very high substrate concentrations (1 mM) GTP is almost ineffective (fig.2).

A third important feature is concerned with the molecular organization of the cyclic GMP PDE, which is stimulated by GTP. It is well known that several molecular forms can be presented by the PDEs resulting from the aggregation of either very similar monomeric subunits or of completely different subunits (as in the case of the calmodulin-dependent PDE [7]) and that it is possible to pass from one molecular organization to another very easily [8,9]. The chromatographic procedure [6] we used to obtain different PDE activities could result in the loss of a GTP binding subunit or in a molecular organization with catalytic activity but unable to bind GTP.

Fig.4 reports that in the presence of high cyclic GMP concentrations IBMX not only loses any inhibitory effect but also reinforces the GTP effect. These results could suggest the existence of at least two IBMX binding sites, with very different affinities, the former responsible for the competitive

mechanism of cyclic GMP and the latter with a very low affinity. The competitive mechanism rules out IBMX from the inhibitory site in the presence of high cyclic GMP concentrations, therefore making it available for the low affinity binding site; this interaction should result either in a direct activation of the enzyme or in an increased affinity of the GTP binding site.

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