GUANOSINE 5',α-β-METHYLENE, TRIPHOSPHATE, A NOVEL GTP ANALOG, CAUSES PERSISTENT ACTIVATION OF ADENYLATE CYCLASE: EVIDENCE AGAINST PYROPHOSPHORYLATION MECHANISM

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SUMMARY: To test the hypothesis that guanine nucleotides activate adenylate cyclase by a covalent mechanism involving pyrophosphorylation of the enzyme, we studied the effect of a novel GTP analog, guanosine 5',α-β-methylene triphosphate ($Gp(CH_2)pp$), with a methylene bond in the α-β-position that is stable to enzymatic hydrolysis. $Gp(CH_2)pp$ was as effective as GTP in stimulating rat reticulocyte adenylate cyclase in the presence of isoproterenol. Previously only guanine nucleotides with modified terminal phosphates such as guanylyl 5'-imidodiphosphate (Gpp(NH)p) were thought capable of causing persistent activation of adenylate cyclase. $Gp(CH_2)pp$, however, caused persistent activation of rat reticulocyte and turkey erythrocyte adenylate cyclase. We conclude that guanine nucleotides do not activate adenylate cyclase by a pyrophosphorylation mechanism and that a modified γ-phosphate is not essential in guanine nucleotides for generation of the irreversibly-activated enzyme state.

Hormonal stimulation of adenylate cyclase in plasma membranes of diverse tissues requires GTP-like nucleotides (1). Studies with GTP analogs nonhydrolyzable at the β-γ-phosphate bonds, such as Gpp(NH)p and $Gpp(CH_2)p$, have provided some insight into the guanine nucleotide effect (1) and have virtually excluded a monophosphorylation mechanism involving the γ-phosphate. The analogs are more effective than GTP in activating adenylate cyclase in the absence of hormones (1,2) and, unlike GTP, allow formation of a persistently activated state of the enzyme (3,4). These and other observations have led to the proposal by Cuatrecasas and his co-workers that GTP activates by a covalent mechanism involving pyrophosphorylation of adenylate cyclase (5).

Abbreviations: Gpp(NH)p - guanylylimidodiphosphate; $Gpp(CH_2)p$ guanylyl 5'-methylene diphosphonate; $Gp(CH_2)pp$ - guanosine 5'-α-β-methylene triphosphate; and $Gp(CH_2)p$ - guanosine 5'-methylene diphosphonate.

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To test this hypothesis directly, we studied the effects of a novel GTP analog, $Gp(CH_2)pp$, with a methylene bond stable to enzymatic hydrolysis in the α-β-position. The studies described here show that $Gp(CH_2)pp$ not only substitutes for GTP in activating adenylate cyclase but that, like Gpp(NH)p, allows development of the persistently activated state. These results make it extremely unlikely that guanine nucleotides activate adenylate cyclase by a pyrophosphorylation mechanism.

MATERIALS AND METHODS

$Gp(CH_2)pp$ and $Gp(CH_2)p$ were synthesized by ICN Chemical and Radioisotope Division following the method of Myers et al. (6) and were chromatographically > 94% pure. Structure was confirmed by NMR spectroscopy. [γ-^{32}P]$Gp(CH_2)pp$ and [γ-^{32}P]GTP were also obtained from ICN. $Gpp(CH_2)p$ was obtained from Miles Laboratories. All other compounds were obtained from previously cited sources (2,4,7).

Turkey erythrocyte and rat reticulocyte membranes were prepared as previously described (2,8). Racemic mixtures of isoproterenol and propranolol were used throughout. Adenylate cyclase assay was performed as previously described (2,4,7) except that in assays using App(NH)p as substrate, the incubation mixture pH was 8.5 and contained no nucleotide-regenerating system. Cyclic AMP was fractionated by the method of Salomon et al. (9) as modified previously (2,4). Inorganic phosphate was measured by the micro-method of Chen, Toribara and Warner (10). $^{32}P_i$ release from [γ-^{32}P]GTP and [γ-^{32}P]$Gp(CH_2)pp$ was measured according to Cassel and Selinger (11).

RESULTS

Using App(NH)p as substrate, stimulation of rat reticulocyte adenylate cyclase by β-adrenergic agents is entirely dependent upon the presence of guanine nucleotides.* This represented an ideal system in which to test the efficacy of $Gp(CH_2)pp$, since GTP is quite effective and a nucleotide regenerating system is unnecessary. $Gp(CH_2)pp$ was as effective as GTP in this system (Table 1). Stimulation by $Gp(CH_2)pp$ was observed at concentrations as low as 0.1 μM and was near maximal by 0.1 mM (not shown).

Since $Gp(CH_2)pp$ was as effective as GTP in rat reticulocyte membranes assayed in the presence of isoproterenol, we tested the effects of the compound under conditions in which GTP itself is ineffective. GTP, at concentrations as high as 0.1 mM, does not cause persistent activation

*Spiegel and Aurbach, unpublished observations.

Table 1. Effect of Guanine Nucleotides on Isoproterenol Stimulation of Rat Reticulocyte Adenylate Cyclase

Guanine Nucleotide Added	None	GTP	$Gp(CH_2)pp$	$Gpp(CH_2)p$	Gpp(NH)p
	Enzyme Activity (pmol cAMP/mg prot./7 min)				
Experiment 1	98±11	1420±178	1250±138	860±92	1950±121
Experiment 2	71±10	824± 24	894± 47	630±61	1310±153

Rat reticulocyte membranes were incubated with 0.125 mM App(NH)p and 0.5 mM isoproterenol in .05 M HEPES buffer (pH 8.5) at 37°. Guanine nucleotides were added at a final concentration of 0.1 mM. The results are the mean (±SD) of quadruplicate determinations from 2 representative experiments.

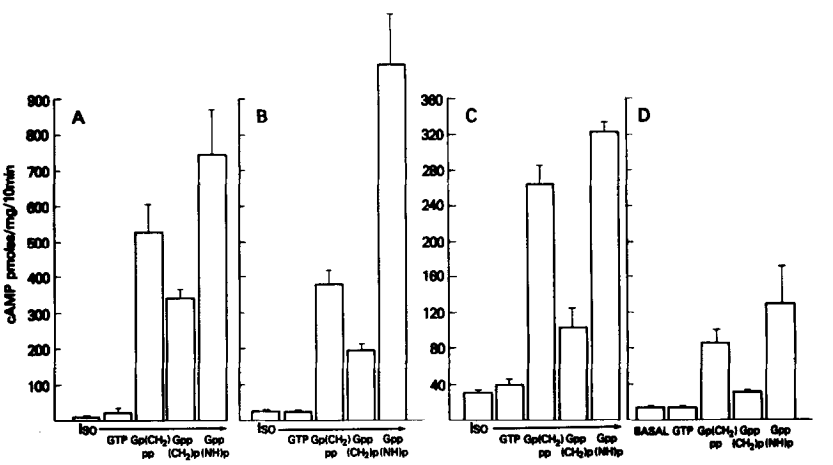

Figure 1A,B,C,D. Effect of guanine nucleotides on adenylate cyclase activity. A and B. Guanine nucleotides (0.1 mM) were added to A) turkey erythrocyte membranes and B) rat reticulocyte membranes in the presence of 0.5 mM isoproterenol and incubated for 30 min at 37°. Thirty µl aliquots of membranes were then assayed for adenylate cyclase activity using ATP (0.125 mM) plus regenerating system, in the presence of 0.5 mM propranolol in a final volume of 70 µl. C and D. Guanine nucleotides (0.1 mM) were added to C) turkey erythrocyte membranes in the presence of 0.5 mM isoproterenol, and D) rat reticulocyte membranes without added isoproterenol, and assayed using 0.125 mM ATP plus regenerating system. Results in A-D are the mean of quadruplicate determinations (± SD) from a representative experiment.

of rat reticulocyte or turkey erythrocyte membranes incubated with isoproterenol (4,8). $Gp(CH_2)pp$, on the other hand, caused development of persistently activated enzyme with turkey erythrocyte (Fig. 1A) and rat reticulocyte (Fig. 1B) membranes. We compared the efficacy of $Gp(CH_2)pp$ with that of

Table 2. Hydrolysis of γ-PO_4 of GTP vs. $Gp(CH_2)pp$

	E. Coli Alk. Phos.	Turkey Eryth. Membranes (nmol/mg/min)	Rat Reticulocyte Membranes
GTP	17	4.4	3.3
$Gp(CH_2)pp$	17	0	0

The reaction mixture contained: GTP (0.1 mM) or $Gp(CH_2)pp$ (0.1 mM), [γ-^{32}P]GTP (25 Ci/mmol) or [γ-^{32}P]$Gp(CH_2)pp$ (52 Ci/mmol), 100-500,000 cpm, and .05 M HEPES buffer pH 8.5, in a final volume of 0.1 ml. Additions were E. Coli alkaline phosphatase (0.02 mg), and turkey erythrocyte or rat reticulocyte membranes (0.05-0.075 mg) to which isoproterenol (0.5 mM) had been added. Following incubation at 37° for 30 min $^{32}P_i$ released was separated from nucleotide-bound phosphate as in Methods (11). The results are the mean of 6-9 determintions from 2 experiments. Individual values differed by <10%.

the β-γ-analogs, $Gpp(CH_2)p$ and Gpp(NH)p, in several systems in which GTP has little effect. In each case the order of activity was Gpp(NH)p > $Gp(CH_2)pp$ > $Gpp(CH_2)p$ >>GTP (Fig. 1A,B,C,D).

In order to assess the lability of the phosphate bonds of the various analogs, we measured inorganic phosphate release following incubation (37° for 30 min in 0.025 M Tris buffer, pH 8.0) of 1 µmol of each guanine nucleotide with or without 0.02 mg of E. Coli alkaline phosphatase (Sigma). Values obtained (mean of duplicates) were corrected for nonenzymatic hydrolysis. With GTP, 1.49 µmol of P_i were released, indicating hydrolysis of both β and γ phosphates, while with $Gp(CH_2)pp$ only the terminal phosphate was cleaved (0.54 µmol P_i released). Gpp(NH)p was a poor substrate (0.12 µmol released) and $Gpp(CH_2)p$ was resistant to cleavage (0.01 µmol released).

The ability of turkey erythrocyte and rat reticulocyte membranes to hydrolyze GTP and $Gp(CH_2)pp$ was then examined, under conditions identical to those in which persistent activation of adenylate cyclase in the membranes was achieved. Both turkey and rat membranes released $^{32}P_i$ from [γ-^{32}P]GTP but were unable to release $^{32}P_i$ from [γ-^{32}P)$Gp(CH_2)pp$ (Table 2). The

ability of E. Coli alkaline phosphatase to cleave the terminal phosphate of both compounds was confirmed with this technique as well (Table 2).

DISCUSSION

The ability of Gpp(NH)p and Gpp(CH_2)p to cause persistent activation of adenylate cyclase led Cuatrecasas and co-workers to suggest that GTP activates adenylate cyclase by pryophosphorylating the enzyme (5). This putative form of the enzyme should be readily pyrophosphorylyzed, deactivating the enzyme. Analogs that form a stable linkage, such as an imidodiphosphoryl enzyme in the case of Gpp(NH)p,would cause irreversible activation. The ability of membrane preparations from several different tissues to cleave the α-β-phosphate bond of Gpp(NH)p made such a mechanism feasible (12). Although we (2) and others (1,13) have shown that membrane-bound Gpp(NH)p remains chromatographically intact following elution from binding sites, this might reflect, as Jacobs and Cuatrecasas proposed, that the major fraction of binding was to sites other than the relevant nucleotide regulatory site (14).

We reasoned that if GTP acts by pyrophosphorylation of adenylate cyclase, a GTP analog with a nonhydrolyzable α-β-phosphate bond should not be capable of substituting for GTP in activating adenylate cyclase and might even function as a competitive inhibitor. We used Gp(CH_2)pp to test this hypothesis directly, since the methylene diphosphonate bond is extremely stable and resists cleavage by all enzymes thus far tested (15).** The ability of Gp(CH_2)pp to substitute effectively for GTP in stimulating rat reticulocyte adenylate cyclase excludes pyrophosphorylation (as well as guanylylation) as a mechanism of action of guanine nucleotides on this enzyme.

An unexpected result of our studies was the ability of Gp(CH_2)pp to cause persistent activation of rat reticulocyte and turkey erythrocyte adenylate cyclase and in general to be more effective than GTP. Gp(CH_2)pp

**Gp(CH_2)pp is unable to be cleaved by guanylate cyclase and inhibits the hydrolysis of GTP by this enzyme (Tsai and Spiegel, unpublished observations).

behaved in this respect, like the β-γ-analogs of GTP but was even more effective than Gpp(CH_2)p.

The greater efficacy of Gpp(NH)p, Gpp(CH_2)p, and guanosine 5'-O-(3-thiotriphosphate) (13) by comparison with GTP has been explained in terms of the resistance of the analogs with a modified γ-phosphate to terminal phosphate cleavage (1). GTP, unlike the β-γ-analogs, can be cleaved by membranes to GDP (1) which binds to the nucleotide regulatory site but is much weaker in activating adenylate cyclase (1).* It was possible that the enhanced efficacy of Gp(CH_2)pp relative to GTP might be explained by stability of Gp(CH_2)p, putatively formed by membrane phosphatase activity. Preliminary experiments testing the effects of Gp(CH_2)p directly in rat reticulocyte membranes suggest, however, that it is at best a very weak stimulator of adenylate cyclase.*

The greater efficacy of Gp(CH_2)pp relative to GTP can be explained by the resistance of Gp(CH_2)pp and not GTP to terminal phosphate cleavage by membrane phosphatases. This was directly demonstrated in turkey erythrocyte and rat reticulocyte membranes. The terminal phosphate of Gp(CH_2)pp, however, was readily cleaved by E. Coli alkaline phosphatase suggesting that it is not universally stable to phosphorolysis at the γ-position. The β-phosphate of Gp(CH_2)pp, as expected, is resistant even to alkaline phosphatase. The terminal phosphate of Gpp(CH_2)p was also resistant to hydrolysis by alkaline phosphatase and that of Gpp(NH)p was cleaved only to a minor degree, in agreement with the results of Yount et al. (15) obtained with the corresponding adenine nucleotides. A modified γ-phosphate is, therefore, not essential in guanine nucleotides for stability of the terminal phosphate and generation of the irreversibly-activated enzyme state.

Our results indicate that Gp(CH_2)pp is a useful analog in investigating the mechanism of the guanine nucleotide effect on adenylate cyclase. Further studies with Gp(CH_2)pp, Gp(CH_2)p and other analogs should provide definitive information concerning the structural requirements for nucleotide regulation

of this complex enzyme. The superiority of $Gp(CH_2)pp$ compared with $Gpp(CH_2)p$, for example, suggests that the position of the modified phosphate bond may be critical in determining intrinsic activity. In addition, $Gp(CH_2)pp$ and $Gp(CH_2)p$ may be useful in investigating the mechanism of action of guanine nucleotides in other processes such as tubulin polymerization (16) and the translocation step of the protein synthesis elongation cycle (17).

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