ACTIVATION OF TURKEY ERYTHROCYTE ADENYLATE CYCLASE AND BLOCKING OF THE CATECHOLAMINE-STIMULATED GTPase BY GUANOSINE 5'-(γ-THIO) TRIPHOSPHATE

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SUMMARY

Experiments with GTP analogues tested how the catecholamine-stimulated GTPase relates to the adenylate cyclase of the turkey erythrocyte membrane. Incubation of the membranes with guanosine 5'-(γ-thio) triphosphate (GTPγS) and isoproterenol followed by washing resulted in persistent activation of the adenylate cyclase and in an inhibition of the catecholamine stimulated GTPase. Incubation of the membranes with GTPγS in the presence of propranolol did not cause activation of the adenylate cyclase nor did it inhibit the catecholamine-stimulated GTPase. The adenylate cyclase activated by guanosine 5'-(β, γ-imino) triphosphate (Gpp(NH)p), rapidly reverted ($t_{\frac{1}{2}}$ = 1 min) to the basal inactive state upon incubation in a GTPase reaction mixture containing isoproterenol. Accordingly, preincubation with Gpp(NH)p and isoproterenol did not cause an inhibition of the catecholamine-stimulated GTPase. These findings suggest that both the catecholamine-stimulated GTPase and the binding of the guanyl nucleotides which activate the adenylate cyclase take place at the same regulatory site.

INTRODUCTION

Studies of adenylate cyclase in various systems have shown that not only are the guanyl nucleotide analogues (Gpp(NH)p and GTPγS) more potent as activators of the enzyme than GTP, but also that unlike GTP, the activation they produce is *quasi*-irreversible (1-5). We have recently found in the turkey erythrocyte membrane a catecholamine-stimulated GTPase. This activity is induced by catecholamines which meet the stereospecific requirements of the β-adrenergic receptor (6). In the present study we have explored the correlation between the GTPase activity and the adenylate cyclase system using the metabolically stable GTP analogues, and demonstrated that persistent activation of the adenylate cyclase coincides with a *quasi*-irreversible inhibition of the catecholamine-stimulated GTPase.

Abbreviations used: App(NH)p and Gpp(NH)p, adenosine and guanosine 5'-(β, γ-imino) triphosphate respectively; GTPγS, guanosine 5'-(γ-thio) triphosphate; NTPase, nucleoside triphosphatase.

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MATERIALS AND METHODS

[α-^{32}P]ATP was obtained from Amersham, England. App(NH)p and Gpp(NH)p were from ICN. GTPγS was the generous gift of Dr. Weiman of the Boehringer Co. and of Dr. Eckstein of the Max-Planck-Institut, Goettingen, Germany. [γ-^{32}P]GTP was prepared according to Glynn and Chappell (7).

Turkey erythrocyte membranes. These were prepared by digestion of the nuclei with DNAase as described for frog erythrocytes (1) with the following modifications: The pH of the Tris-buffer used for lysis and for the ensuing washes was 7.9 at 23°C, and β-mercaptoethanol (2 mM) was used instead of dithiothreitol. The membranes were stored in liquid nitrogen and thawed on the day of the experiment. Freezing and thawing appeared to enhance the effect of catecholamines on GTP hydrolysis.

GTPase assay. GTPase activity was measured by liberation of ^{32}Pi from [γ-^{32}P]GTP essentially as previously described (6). The reaction mixture of the final volume of 0.1 ml contained 0.25 μM [γ-^{32}P]GTP (2-20 mCi/μmole), 6 mM $MgCl_2$, 0.5 mM App(NH)p, 0.1 mM ATP, 3 mM creatine phosphate, 3 units of creatine phosphokinase, 2 mM β-mercaptoethanol, 0.1 mM EGTA, and 50 mM imidazole-HCl buffer, pH 6.7. The reaction was initiated by the addition of the membranes (20-40 μg protein) and terminated after 10 min at 37°C by adding 0.5 ml of 5% charcoal (Norit A) suspension in an ice cold NaPi buffer (20 mM, pH 7.0). After 5 min in the cold, the suspension was centrifuged (1,000 xg for 5 min) and radioactivity determined on aliquots of the supernatant. The ^{32}Pi found in systems which had no membranes (2-3% from the [γ-^{32}P]GTP in the assay system) was deducted from the values found in systems containing membranes. The catecholamine-stimulated GTPase activity was calculated directly by subtracting the amount of ^{32}Pi counts liberated in a tube without catecholamine from the amount liberated in an identical reaction mixture but containing catecholamine (50 μM-dl-isoproterenol). The basal GTPase was calculated after subtracting the counts of ^{32}Pi formed upon isotope dilution with unlabeled GTP (30 μM final concentration) from the counts of ^{32}Pi formed in the absence of unlabeled GTP (6). Assays were performed in triplicate.

Adenylate cyclase assay. Activity was assayed according to the method of Salomon *et al.* (8). The reaction mixture contained 0.3 mM [α-^{32}P]ATP (20-60 cpm/pmole), 6 mM $MgCl_2$, 1 mM cyclic AMP, 12 mM creatine phosphate, 3 units of creatine phosphokinase, 0.2 mM EGTA, 2 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 7.5. Final volume was 0.1 ml, and reaction time was 10 min at 37°C.

Activation of the adenylate cyclase by guanyl nucleotide analogues. Turkey erythrocyte membranes (1-2 mg protein/ml) were suspended in a medium containing 1 mM $MgCl_2$, 2 mM β-mercaptoethanol, and 20 mM Tris-HCl buffer, pH 7.5, and were incubated for 5 min at 30°C in the presence of isoproterenol (20 μM) and either 20 μM Gpp(NH)p or 0.2 μM GTPγS. The control systems contained propranolol (1 μM) instead of isoproterenol. At the end of the incubation the membranes were washed four times in the cold by centrifugation (10,000 xg for 10 min) and resuspended in 10 volumes of medium. Protein was determined according to the method of Lowry *et al.* (9) using bovine serum albumin as standard.

RESULTS

We have recently reported that the turkey erythrocyte membrane shows a basal and a catecholamine-stimulated GTPase activity, both of which

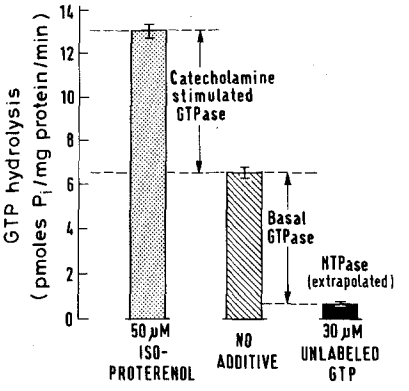


Fig. I: *Activities of the specific GTPases in turkey erythrocyte membranes.*

The basal and the catecholamine stimulated GTPase activities are calculated as described under Materials and Methods. The data shown in the right column represent the contribution of low affinity nucleoside triphosphatases to hydrolysis of GTP at a concentration of 0.25 μM. This contribution was estimated by addition of unlabeled GTP (30 μM) and converting the counts of the ^{32}Pi formed into pmoles, using the specific radioactivity of the assay systen containing the standard [γ-^{32}P]GTP concentration (0.25 μM). This calculation is based on the finding that the NTPases have low affinity for GTP (Km ≈ 200 μM) (6). Therefore, the increase in GTP concentration from 0.25 μM to 30 μM causes a proportional increase in the NTPase activity. However, as specific radioactivity is proportionately decreased by adding the unlabeled GTP, the two factors cancel each other, so that the amount of counts of ^{32}Pi liberated by the NTPases remained unchanged.

have high affinity for GTP (Km ≈ 0.1 μM) (6). In the present study, membranes were prepared by lysis of turkey erythrocytes at pH 7.9, and the GTPase assay contained a higher App(NH)p concentration than previously employed in order to inhibit the non-specific nucleoside triphosphates more effectively (*c.f.*, ref. 6). Under these conditions, the basal GTPase accounted for 90% of the total GTP hydrolysis, and the addition of a catecholamine agonist caused a two-fold increase in the hydrolysis (Fig. 1).

In view of the persistent activation of the adenylate cyclase by metabolically stable GTP analogues and the stable binding of the analogue to the regulatory site (10), it has been conceived that the GTP analogues might block the GTPase at the regulatory site. To test this hypothesis, the GTP analogues Gpp(NH)p and GTPγS, which were found to be the most potent activators of the pigeon erythrocyte adenylate cyclase (2), were used. Incubation of turkey erythrocyte membranes with GTPγS (0.2 μM) and isoproterenol followed by washing, produced efficient activation of the adenylate cyclase as well as an inhibition of the catecholamine-stimulat-

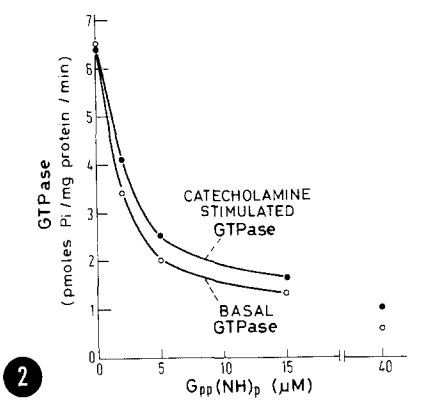
Table I: *Effects of treatment with Gpp(NH)p and GTPγS on GTPase and adenylate cyclase activities.*

Membranes (1.5 mg/ml) were preincubated with the GTP analogue and then washed thoroughly, as described under Materials and Methods. Membrane concentration in the assays was 0.35 mg/ml. Basal GTPase was calculated after subtraction of the NTPase activity (0.3 - 0.6 pmoles/mg/min), as described in Fig. 1. Data are mean ± S.D. (n = 3). The experiment shown was repeated five times with essentially the same results.

Preincubation with	GTPase activity				Adenylate cyclase activity
	No additive	Isoproterenol	Basal GTPase	Catecholamine stimulated GTPase	
	pmoles Pi/mg protein/min				pmoles cAMP/mg protein/min
No additive	6.6 ± 0.1	13.2 ± 0.1	6.0	6.6	6
Propranolol	7.0 ± 0.1	12.9 ± 0.3	6.4	5.9	5
Isoproterenol	6.2 ± 0.1	12.4 ± 0.4	5.6	6.2	7
Gpp(NH)p + Propranolol	5.1 ± 0.2	11.0 ± 0.4	4.5	5.9	9
Gpp(NH)p + Isoproterenol*	4.1 ± 0.1	8.1 ± 0.2	3.8	4.0	247
GTPγS + Propranolol	4.9 ± 0.2	9.8 ± 0.2	4.5	4.9	14
GTPγS + Isoproterenol*	5.1 ± 0.2	6.5 ± 0.1	4.8	1.4	397

* Adenylate cyclase activity, when assayed in the presence of 50 μM isoproterenol plus 50 μM of either Gpp(NH)p or GTPγS, was 350 and 418 pmoles mg^{-1} min^{-1}, respectively.

ed GTPase by 75% (Table I). However, preincubation with GTPγS in the presence of the β-adrenergic blocker propranolol did not cause activation of the adenylate cyclase and neither did it inhibit the catecholamine-stimulated GTPase. In contrast to GTPγS, preincubation with Gpp(NH)p in the presence of either isoproterenol or propranolol had little effect on the catecholamine-stimulated GTPase (Table I). Preincubation with either Gpp(NH)p or GTPγS resulted in a small inhibition of the basal GTPase, which was not dependent on the presence of isoproterenol. Although preincubation of the membranes with Gpp(NH)p followed by washing did not inhibit the GTPase (Table I), Gpp(NH)p did inhibit both the basal and the



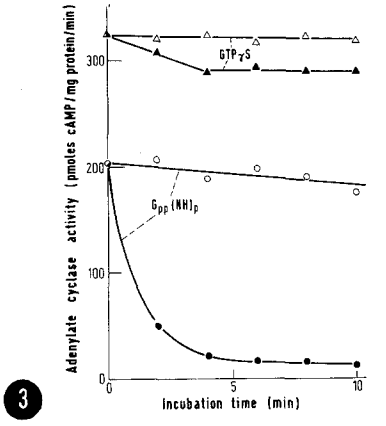


Fig. 2: *Inhibition of the GTPase activities by various Gpp(NH)p concentrations.*

The concentrations of Gpp(NH)p indicated were added to the standard GTPase assay.

Fig. 3: *Effects of isoproterenol on adenylate cyclase activity in membranes pretreated with Gpp(NH)p and GTPγS.*

Activation of the adenylate cyclase by GTP analogues and isoproterenol was as described in Materials and Methods. The washed membranes (0.8 mg/ml) were incubated at 37°C in GTPase assay medium which contained either 50 μM isoproterenol (closed symbols) or no addition (open symbols). At the times indicated on the abscissa, 20 μl aliquots were removed to an adenylate cyclase assay system containing 50 μM propranolol, and enzyme activity was determined as described in Materials and Methods.

catecholamine-stimulated GTPase when present in the GTPase assay (Fig. 2, K_I = 1 μM). A similar Gpp(NH)p concentration half maximally stimulated the turkey erythrocyte adenylate cyclase (10).

The effect of the GTPase assay components on the persistence of the guanyl nucleotide activated adenylate cyclase is illustrated in Fig. 3. Incubation of the Gpp(NH)p activated adenylate cyclase in the GTPase reaction mixture containing isoproterenol, resulted in a rapid decline of enzyme activity, whereas when activated by GTPγS, adenylate cyclase persisted in full activity under the same conditions. Thus, the ability of the guanyl nucleotide analogue to block the isoporterenol stimulated GTPase (Table I) reflects whether or not the guanyl nucleotide activated adenylate cyclase remains active in the GTPase assay system containing isoproterenol (Fig. 3).

DISCUSSION

The differences in the persistence of the effect of Gpp(NH)p and of

GTPγS on the adenylate cyclase system is probably quantitative rather than qualitative. The K_{diss} values in activation of the adenylate cyclase were for GTPγS 10^{-8} M, and for Gpp(NH)p 5 x 10^{-7} M. Since half of the Gpp(NH)p is released from the regulatory site within 1 min (Fig. 3 and ref. 10), a dissociation of GTPγS at 1/50 of the rate would enable this analogue to remain bound to the regulatory site for about 1 hr.

In view of the dependence of a persistent activation of turkey erythrocyte adenylate cyclase by GTP analogues on the presence of a β-adrenergic agonist, the finding of an isoproterenol-dependent blocking of catecholamine-stimulated GTPase by GTPγS (Table I, exp. 6-7) supports our thesis that the guanyl nucleotide regulatory site of the adenylate cyclase is identical to the GTP binding site of the catecholamine-stimulated GTPase. The similar affinity and specificity of the GTPase and the adenylate cyclase to guanyl nucleotides and to β-adrenergic agonists and antagonists (6) are consistent with the identification of GTPase as part of the adenylate cyclase system. The hydrolysis of GTP at the regulatory site, terminates activation of the adenylate cyclase (6). The GTP analogue, however, is not hydrolyzed by the GTPase (10), and thus brings about persistent activation of the adenylate cyclase.

In contrast to the catecholamine-stimulated GTPase, the basal GTPase was not blocked upon activation of the adenylate cyclase by GTPγS and isoproterenol (Table I). This finding is in accord with our previous conclusion that the basal and the catecholamine stimulated GTPases are two different entitities (6) and suggests that the basal GTPase is not part of the activated adenylate cyclase complex.

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