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CATECHOLAMINE-STIMULATED GTPase ACTIVITY IN TURKEY ERYTHROCYTE MEMBRANES

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Summary

Determination of specific GTPase (EC 3.6.1.—) activity in turkey erythrocyte membranes was achieved using low concentration of GTP (0.25 μM), inhibition of nonspecific nucleoside triphosphatases by adenosine 5′(β,γ-imino)-triphosphate(App(NH)p) and suppression of the transfer of γ-^{32}P from GTP to ADP with an ATP regeneration system. Under these conditions catecholamines caused a 30—70% increase in GTP hydrolysis. The stimulation of GTPase activity by catecholamines required the presence of Mg^{2+} or Mn^{2+}. Different batches of membranes revealed the following specific activities (pmol $^{32}P_i$/mg protein min): basal GTPase (determined in the absence of catecholamine), 6—11; catecholamine-stimulated GTPase, 3—7; and residual non-specific NTPase, 3—5. The stimulation of GTPase activity by catecholamines fulfilled the stereospecific requirements of the β-adrenergic receptor, and was inhibited by propranolol. The concentrations of DL-isoproterenol which half-maximally activated the GTPase and adenylate cyclase were 1 and 1.2 μM, respectively.

The following findings indicate that the catecholamine-stimulated GTPase is independent of the catalytic production of cyclic AMP by the adenylate cyclase. Addition of cyclic AMP to the GTPase assay did not change the rate of GTP hydrolysis. Furthermore, treatment of the membrane with *N*-ethylmaleimide (MalNEt) at 0°C which caused 98% inhibition of the adenylate cyclase, had no effect on the catecholamine-stimulated GTPase.

The affinity and specificity for GTP in the GTPase reactions are similar to those previously reported for the stimulation of the adenylate cyclase. The apparent K_m for GTP in the basal and the catecholamine-stimulated GTPase reaction was 0.1 μM. These GTPase activities were inhibited by ITP but not by CTP and UTP.

The abbreviations used are: Gpp(CH_2)p, guanosine 5′-(β,γ-methylene) triphosphate; App(NH)p and Gpp(NH)p, adenosine and guanosine 5′-(β,γ-imino)triphosphate, respectively; GTPγS, guanosine 5′-(γ-thiotriphosphate); cyclic AMP, adenosine 3′,5′-monophosphate; MalNEt, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycolbis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; NTPase, non-specific nucleoside triphosphatase.

It is proposed that a catecholamine-stimulated GTPase is a component of the turkey erythrocyte adenylate cyclase system.

Introduction

Guanylnucleotides which were initially shown to activate hepatic adenylate cyclase [1,2] are now recognized as common effectors of this enzyme in a variety of eukaryotic cells [3—6]. More recently the introduction of GTP analogues, poorly hydrolyzed by nucleoside triphosphatases, has greatly facilitated the study of the guanylnucleotide's role in the adenylate cyclase system. These analogues, guanosine 5′(β,γ-imino)triphosphate, (Gpp(NH)p), guanosine 5′-(γ-thiotriphosphate)(GTPγS) and guanosine 5′(β,γ-methylene)triphosphate (Gpp(CH_2)p) by themselves activate the adenylate cyclase. However, maximal activation of the adenylate cyclase is obtained when the analogue and hormone act together on the membrane [6—8]. In frog and avian erythrocytes, activation of the adenylate cyclase by the GTP analogues and epinephrine is quasi-irreversible and following a short incubation period, neither the GTP analogue, nor the hormone are required to maintain activity [6,7,9]. The stimulation of the adenylate cyclase by GTP and the hormone, however, is completely reversible and enzyme activity requires the continuous presence of both GTP and the hormone. Since GTP competitively inhibits the activation of the adenylate cyclase by Gpp(NH)p it is conceivable that GTP and its analogues act on the same regulatory site [10,11]. The difference between GTP and its analogues in activation of the adenylate cyclase has led several investigators to suggest that the adenylate cyclase system has also a GTPase (EC 3.6.1. —) activity [4,6]. Direct study of GTPase activity was carried out by Pfeuffer and Helmreich [6] who purified a guanylnucleotide-binding protein from pigeon erythrocyte membrane. These investigators reported that in the intact membrane the ratio of GTPase to ATPase activity is 0.8. Following a 40-fold purification, however, this ratio rose only to 2.0. Salomon and Rodbell [12] reported that GTP tightly bound to liver and fat cell membrane is degraded to GDP and GMP. Despite these observations direct evidence for the relationship of these activities to the adenylate cyclase system could not be demonstrated.

In the present work an analysis of the factors which influence the hydrolysis of GTP by turkey erythrocyte membranes, led to the development of a specific assay of GTPase activity. It was found that catecholamines increase the GTPase activity through their action on the β-adrenergic receptor.

Materials and Methods

[α-^{32}P]ATP was obtained from the Radiochemical Centre, Amersham, England. Carrier-free $H_3^{32}PO_4$ was obtained from the Nuclear Center, Negev, Israel. ATP, GTP, CTP, ADP, GDP, cyclic AMP, creatine phosphate, creatine phosphokinase, L-isoproterenol, L-norepinephrine, L-phenylephrine, MalNEt and *p*-chloromercuribenzoic acid were obtained from Sigma Chemical Corp., DL-isoproterenol from Mann Research Lab., D- and L-epinephrine from K and K Laboratories, DL-propranolol and Lubrol PX from I.C.I. Catechol from B.D.H.

Trypsin and chymotrypsin were from Worthington. Phospholipase *C* (*B. cereus*) from Makor Chemicals, Israel. App(NH)p from I.C.N. ITP from Waldhof, UTP from Boehringer. 3-isobutyl-1-methylxanthine from Aldrich. D-propranolol was kindly given by I.C.I. Ltd., U.K. [γ-^{32}P]GTP was prepared by the method of Glynn and Chappell [13].

Turkey erythrocyte membranes

These were prepared as described for frog erythrocytes [7] by a modification of the procedure of Rosen and Rosen [14], except that β-mercaptoethanol (2 mM) was employed instead of dithiothreitol. Differenct batches of membrane preparation showed catecholamine stimulated adenylate cyclase activity (pmol cyclic AMP/mg protein per min) of 50—250. Membranes were stored in liquid nitrogen for 2—3 months without a significant loss of adenylate cyclase activity. Membrane preparations which were thawed on the day of the experiments were used for all enzyme assays.

GTPase assay

The following standard conditions were used to measure liberation of $^{32}P_i$ from [γ-^{32}P]GTP. The assay system contained 0.25 μM [γ-^{32}P]GTP (2—10 mCi/μmol), 5 mM $MgCl_2$, 0.2 mM App(NH)p, 0.1 mM ATP, 2 mM creatine phosphate, 3 units of creatine phosphokinase, 2 mM β-mercaptoethanol, 0.1 mM EGTA, 50 mM imidazole · HCl buffer, pH 6.7. The final volume was 0.1 ml. Any changes from these standard conditions are indicated in individual experiments. The reaction was initiated by the addition of erythrocyte membranes (10—40 μg protein) to the mixture at 37°C and was terminated after 10-min incubation by the addition of 0.1 ml 2.5% SDS solution. The $^{32}P_i$ formed was separated from nucleotide-bound phosphate on a small charcoal column (0.7 × 7 cm). This was made up of a filtering layer of 0.2 g celite 545, overlaid with a mixture of 0.2 g celite 545 and 25 mg acid washed charcoal (Norit A). 1 ml phosphate buffer (50 mM, pH 7.0) was added to each reaction tube and the mixture was passed through the column. The tube was rewashed with an additional 1 ml buffer which was placed on the column and elution of $^{32}P_i$ was completed by a further addition of 1.5 ml buffer. Eluates were collected directly into scintillation vials and counted in a scintillation spectrometer using Cerenkow radiation. The recovery of $^{32}P_i$ was 95—100%. The preparations of [γ-^{32}P]GTP contained 2—6% P_i. This blank was subtracted from the determinations. GTPase assays were performed in triplicate.

Adenylate cyclase assay

Assays were performed in duplicate. The reaction mixture contained 0.3 mM [α-^{32}P]ATP (20—100 cpm/mol), 6 mM $MgCl_2$, 1 mM cyclic AMP, 12 mM creatine phosphate, 3 units of creatine phosphokinase, 0.1 mM EGTA, 1 mM β-mercaptoethanol, 50 mM imidazole · HCl buffer pH 6.7. The final volume was 0.1 ml. The reaction was initiated by the addition of membranes (20—200 μg). Following incubation for 10 min at 37°C the reaction was terminated by the addition of 100 μl stopping solution containing 2% SDS 40 mM ATP. Cyclic AMP was separated and determined by the method of Salomon et al. [15].

Chromatography

Nucleotides were separated by chromatography on polyehtylenimine cellulose plates (Polygram Cell 300, Machery and Nagel Co., Duren, Germany). Adenine and guanine nucleotides were separated using successively: 0.5 M $(NH_4)_2SO_4$ (running distance 4 cm) and 0.7 M $(NH_4)_2SO_4$ (running distance 10 cm) [16]. The ultraviolet absorbing areas were cut out, placed in scintillation vials containing water, and radioactivity was determined using Cerenkow radiation. The R_f values were: GTP, 0.19: ATP, 0.29: App(NH)p, 0.35; ADP, 0.43. ITP and UTP (2 μmol per plate) were separated from GTP using 2 M LiCl as developer and running distance of 15 cm. The R_f values were: UTP, 0.60; ITP, 0.53; GTP, 0.30. LiCl was then removed by soaking the plates in methanol. The nucleotides were transferred to paper [17] using 2 M NH_4HCO_3 and this salt was later removed from the paper by evaporation overnight under reduced pressure. Finally the nucleotides were extracted in water. Phosphate and pyrophosphate in the GTPase assay were detected in charcoal column eluates by chromatography on Whatman 3 mm paper. The solvent system was: isopropanol, 75 ml/H_2O, 25 ml/trichloroacetic acid, 5 g/conc. NH_4OH, 0.3 ml. The areas corresponding to marker spots were cut out and radioactivity determined in toluene-based scintillation liquid. Protein was measured by the method of Lowry et al. using bovine serum albumin as a standard [18]. All the experiments were repeated at least three times and a representative example is shown.

Results

The design of a GTPase assay in turkey erythrocyte membranes

The determination of a nucleoside triphosphatase spcific for GTP in a multifunctional system, such as a membrane, requires an assay in which there is a maximal suppression of the non-specific nucleoside triphosphatase activity. Indeed the use of a low concentration of GTP (0.25 μM) and the addition of App(NH)p, an inhibitor of a number of ATPases [1,19] greatly decreased the rate of GTP hydrolysis by non-specific NTPases (Table I). Chromatographic analysis revealed however that the membrane caused about an equal distribution of ^{32}P in GTP and adenine nucleotides (Table II). Apparently the membrane con-

TABLE I

HYDROLYSIS OF GTP BY TURKEY ERYTHROCYTE MEMBRANES

Membranes were incubated in the presence of 0.25 μM [γ-^{32}P]GTP, 5 mM $MgCl_2$, 50 mM imidazole · HCl buffer (pH 6.7) and other additions as indicated. The ATP regeneration system was 2 mM creatine phosphate and 3 units of creatine phosphokinase. The data are the mean ± half range of duplicate determinations

Additions	[γ^{32}-P]GTP hydrolysis (pmol/mg protein per min)
None	1200 ± 50
0.5 mM App(NH)p	19.4 ± 0.2
0.5 mM App(NH)p + ATP regeneration system	9.5 ± 0.2
0.5 mM App(NH)p + ATP regeneration system + 0.1 mM ATP	7.4 ± 0.1

TABLE II

THE EFFECT OF ATP REGENERATION SYSTEM ON THE TRANSFER OF ^{32}P FROM [γ-^{32}P]GTP TO ADENINE NUCLEOTIDES

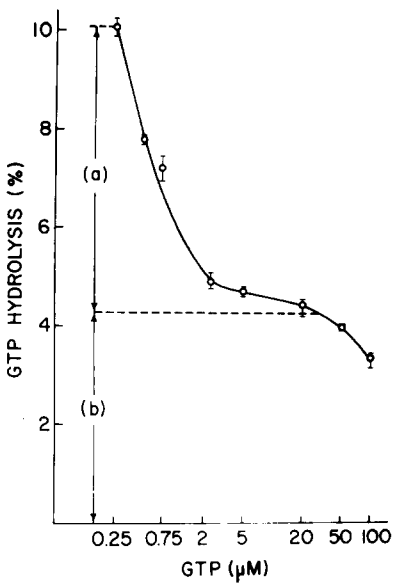
The reaction mixture contained 0.25 μM [γ-^{32}P]GTP, 5 mM $MgCl_2$, 0.5 mM App(NH)p, 50 mM imidazole · HCl (pH 6.7) and other additions as indicated. The ATP regeneration system was 2 mM creatine phosphate and 3 units of creatine phosphokinase. In order to obtain a similar liberation of $^{32}P_i$, in presence and absence of NTP regeneration system, the concentrations of membranes used were 0.5 mg/ml and 0.25 mg/ml, respectively (see Table I). Where indicated, the concentration of ATP was 0.1 mM; isoproterenol was 0.05 mM. The reactions were terminated by immersion in a boiling water bath for 3 min, followed by centrifugation. Aliquots from the supernatant were loaded on PEI-cellulose plates and chromatography was carried out as described under Materials and Methods. All values are cpm.

Nucleotide	Without regeneration system	With regeneration system			Without membranes
		No addition	ATP	ATP + isoproterenol	
ADP	585	43	53	38	30
ATP	642	95	73	62	44
GTP	1021	2158	2596	2389	2906
Total ^{32}P in nucleotides	2248	2296	2722	2489	2980

tains both a nucleoside diphosphokinase and an adenylate kinase which, in the presence of traces of ADP lead to redistribution of radioactivity among guanine and adenine nucleotides.

To prevent the transfer reaction a nucleoside triphosphate regeneration system was added to the assay. This addition acts as a "trap" for nucleoside diphosphates and caused 85—90% suppression of the appearance of ^{32}P in adenine nucleotide and a 50% decrease in the liberation of $^{32}P_i$ in the GTPase assay. When ATP (0.1 mM) was also added to the assay, the suppression of the transfer reaction reached 95% and the liberation of $^{32}P_i$ was decreased by 60% (Tables I and II). The [^{32}P]ATP which accumulates in the absence of NTP regeneration system is probably rapidly hydrolyzed by specific ATPases and this leads to an increased liberation of $^{32}P_i$. It should be noted that the NTP regeneration system could not have significantly suppressed the liberation of $^{32}P_i$ from GTP through isotope dilution. This is because conditions were chosen whereby total hydrolysis of GTP did not exceed 10—20%.

In the present assay a high affinity, specific GTPase (K_m = 0.1 μM) caused about 60% of the total [γ-^{32}P]GTP hydrolysis. Liberation of $^{32}P_i$ by this enzyme is effectively inhibited by addition of unlabeled GTP (Fig. 1). In the presence of excess unlabeled GTP, the $^{32}P_i$ liberated is due to the activity of low affinity NTPases ($K_m \approx 200$ μM) which rises linearly with increasing GTP concentration. The high-affinity specific GTPase (referred to as basal GTPase) is thus calculated as follows: Activity in the presence of 0.25 μM [γ-^{32}P]GTP minus activity in the presence of both 0.25 μM [γ-^{32}P]GTP and 30 μM unlabeled GTP. The non-specific NTPase could be further inhibited by increasing the concentration of App(NH)p in the assay. For the sake of economy, however, 0.2 mM App(NH)p was routinely used.



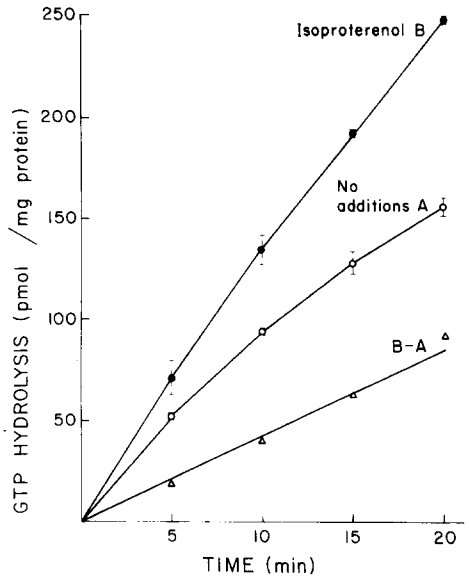


Fig. 1. Isotope dilution curve of GTP hydrolysis. Various concentrations of unlabeled GTP were added to the standard assay system to give the indicated total concentration of GTP. Membrane concentration was 0.25 mg/ml. The decrease in $^{32}P_i$ liberation from [γ-^{32}P]GTP, caused by the addition of 3 nmol of unlabeled GTP is marked as (a) and the residual $^{32}P_i$ liberation is marked (b). The horizontal bars indicate the upper and lower deviations from the mean of triplicate determinations.

Fig. 2. Time course of GTP hydrolysis in the presence and absence of isoproterenol. Membranes (0.2 mg/ml) were incubated as described in Materials and Methods, but in a final volume of 1 ml. At the times indicated. aliquots of 100 μl were added to 100 μl of SDS (2.5%). Where added, isoproterenol was 50 μM. The horizontal bars indicate the deviations from the mean duplicate determinations.

The effect of isoproterenol on GTP hydrolysis

Addition of isoproterenol to the assay system caused 30—70% increase in the liberation of $^{32}P_i$ from GTP. Using different batches of membranes the increment in GTPase activity varied between 3 and 7 pmol $mg^{-1} \cdot min^{-1}$. The catecholamine-stimulated GTPase reaction routinely determined in the presence of DL-isoproterenol was linear for 20 min at 37°C (Fig. 2). There was also a linear relationship between membrane protein and GTP hydrolysis in the range of 10—40 μg of membrane protein incubated for 10 min (data not shown). It should be pointed out that isoproterenol did not increase the amount of $^{32}P_i$ transferred from GTP to adenine nucleotides (Table II).

Both in the presence and absence of isoproterenol, the reaction product isolated from the charcoal column, was identified by paper chromatography as $^{32}P_i$. Less than 5% of the counts were found in the area corresponding to the migration of pyrophosphate. The same results were obtained whether or not pyrophosphate (2 mM) was added to the GTPase reaction mixture. Had labeled pyrophosphate been formed, it would have been detected, since only 5% of the added pyrophosphate was hydrolyzed under these conditions. The presence of the nucleoside triphosphate regeneration system precluded the measurement of GDP.

The β-adrenergic specificity of the catecholamine-stimulated GTPase

Catecholamines in the L-configuration which activate the adenylate cyclase through the β-adrenergic receptor, were also potent activators of FTP hydrolysis. On the other hand, D-epinephrine, dopamine and L-phenylephrine which do not effectively stimulate the turkey erythrocyte adenylate cyclase [20] had little, if any effect on the GTPase reaction (Table III). The concentration of DL-isoproterenol which half-maximally activated the catecholamine stimulated GTPase reaction was approx. 1 μM (Fig. 3). This value is close to the concentration of isoproterenol (1.2 μM) which half-maximally activated the adenylate cyclase at pH 6.7 (data not shown). Since addition of cyclic AMP in the presence of a phosphodiesterase inhibitor had no effect on the hydrolysis of GTP it is unlikely that the effect of isoproterenol on the GTPase activity is caused indirectly through the action of cyclic AMP (Table IV). The β-adrenergic blocker DL-propranolol, had no effect on GTP hydrolysis in the absence of catecholamine, although, when present together with isoproterenol, it completely inhibited the stimulation of the GTPase activity. The D-isomer of propranolol failed to inhibit the catecholamine-stimulated GTPase (Table IV). As in other eukaryotic cells fluoride activates the adenylate cyclase system of turkey erythrocyte membranes without involvement of the β-adrenergic receptor. In contrast to catecholamines NaF decreased GTP hydrolysis and also caused a partial inhibition of the isoproterenol stimulated GTPase (Table IV). Thus, activation of the adenylate cyclase system does not necessarily lead to an increased hydrolysis of GTP.

The affinity and specificity of the GTPase reactions

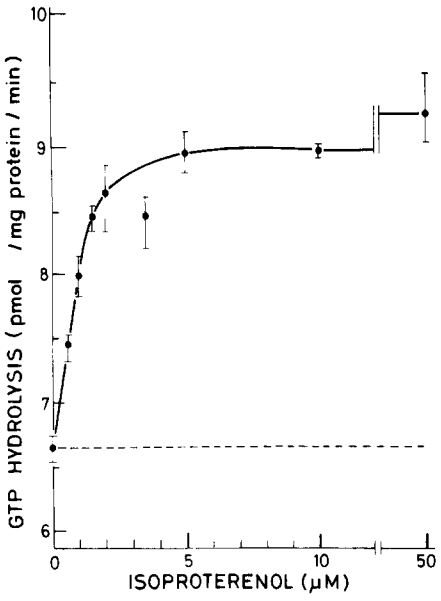
The rate of GTP hydrolysis at various concentrations of GTP, both in the presence and absence of isoproterenol, is depicted in Fig. 4. At the concentration of GTP employed, the membrane non-specific NTPases were not saturated, and thus, the curve did not reach a plateau. On the other hand, the curve of the increment in GTP hydrolysis caused by isoproterenol, reached a plateau and a half-maximal increase in GTP hydrolysis was obtained at 0.1 μM GTP. Subtraction of the non-specific GTP hydrolysis from the total GTP hydrolyzed in the absence of hormone gives the "basal GTPase" (cf. Fig. 1). This

TALBE III

SPECIFICITY OF CATECHOLAMINES IN STIMULATION OF GTP HYDROLYSIS

Membranes (0.19 mg/ml) were incubated as described in Materials and Methods. The data are the mean ± half the range of triplicate determinations.

Addition	$^{32}P_i$ liberated (pmol/mg protein per min)	Increment in $^{32}P_i$ liberated (pmol/mg protein per min)
No addition	10.3 ± 1.2	
(1) 0.1 mM L-isoproterenol	15.7 ± 0.1	5.4
(2) 0.1 mM L-norepinephrine	14.8 ± 0.6	4.5
(3) 0.1 mM L-epinephrine	14.7 ± 0.3	4.4
(4) 0.1 mM L-epinephrine	10.7 ± 0.7	0.4
(5) 0.1 mM dopamine	11.2 ± 0.7	0.9
(6) 0.5 mM catechol	10.0 ± 0.2	—0.3
(7) 2.0 mM L-phenylephrine	10.2 ± 0.1	—0.1



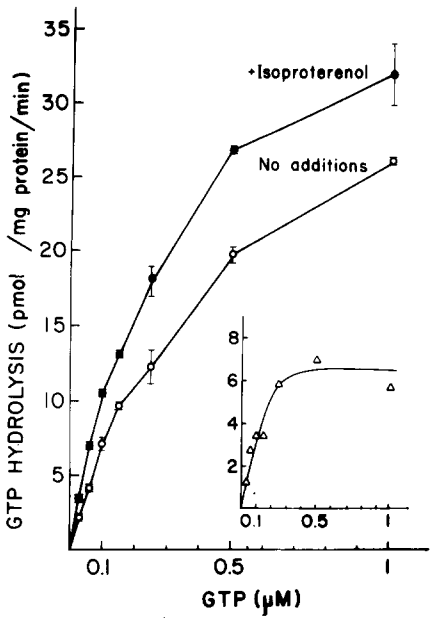


Fig. 3. The rate of GTP hydrolysis at various DL-isoproterenol concentrations. The concentrations of App(NH)p in the GTPase assay was 0.5 mM. Membrane concentration was 0.4 mg/ml. The horizontal bars indicate the upper and lower deviations from the mean of triplicate determinations.

Fig. 4. The rate of GTP hydrolysis at various GTP concentrations. GTP hydrolysis was determined in the standard assay with varying concentrations of GTP. Membrane concentration was 0.16 mg/ml. ○———○ no additions; ●———● isoproterenol (50 μM); △———△ increment in GTPase activity caused by isoproterenol. The horizontal bars indicate the upper and lower deviations from the mean of triplicate determinations.

activity reached saturation and the half-maximal rate of GTP hydrolysis was obtained at 0.1 μM GTP (data not shown). A similar concentration of GTP was found to activate half-maximally the adenylate cyclase [1,5,6]. It has been previously reported that various nucleoside triphosphates can substitute for GTP in activation of hormone-stimulated adenylate cyclases [5,21,22]. In turkey erythrocyte membranes both ITP and UTP were found to activate the catecholamine-stimulated adenylate cyclase; however, much higher concentrations of these nucleotides as compared with GTP were required to produce a half-maximal response [5]. Working with purified nucleotides we confirmed that ITP activates the adenylate cyclase and found that it also competes with GTP in both the basal and the hormone-stimulated GTPase ($K_i \approx 10$ μM) reactions. Purified UTP however had little effect either on the adenylate cyclase or on the GTPase reactions (Table V). Thin layer chromatography of commercial preparation of UTP revealed an ultraviolet-absorbing contaminant which moved on the chromatogram like GTP. This material was highly active in stimulation of the adenylate cyclase and efficiently competed with GTP in the GTPase reactions. It is thus likely that effects on adenylate cyclase found with a commercial preparation of UTP are due to its contamination with GTP.

A ten-fold increase in the concentration of App(NH)p in the reaction mix-

TABLE IV

EFFECTS OF ACTIVATORS AND INHIBITORS OF THE ADENYLATE CYCLASE ON GTP HYDROLYSIS

The concentration of membranes used was 0.4 mg/ml. The data are the mean ± half the range of triplicate determinations.

Addition	$^{32}P_i$ liberated (pmol/mg protein per min)	Increment in $^{32}P_i$ liberation (pmol/mg protein per min)
(1) No additions	8.3 ± 0.3	
(2) 20 μM DL-isoproterenol	11.8 ± 0.1	3.5
(3) 3 μM DL-propranolol	8.4 ± 0.1	0.1
(4) 20 μM DL-isoproterenol + 3 μM DL-propranolol	8.6 ± 0.1	0.3
(5) 20 μM DL-isoproterenol + 3 μM D-propranolol	11.3 ± 0.5	3.0
(6) 10 mM NaF	7.0 ± 0.1	—1.3
(7) 10 mM NaF + 20 μM DL-isoproterenol	9.7 ± 0.1	2.7 *
(8) 0.3 mM 3-isobutyl-1-methylxanthine	8.2 ± 0.4	—0.1
(9) 0.3 mM 3-isobutyl-1-methylxanthine + 10 μM cyclic AMP	8.3 ± 0.1	0.0

* Increment relative to (6).

ture (0.1—1 mM) had no effect on either the basal or the catecholamine-stimulated GTPases (data not shown). It is thus evident that over this concentration range, App(NH)p does not compete with GTP in the GTPase reactions.

Properties of the "basal" and the catecholamine-stimulated GTPase reactions

The catecholamine-stimulated GTP hydrolysis had an optimum of activity at pH 6.5 and was almost completely abolished below pH 5.0. The rate of the "basal" GTPase determined in the absence of isoproterenol, remained constant between pH 3.5 and 8.5 and was abolished only at pH 3.0 (data not shown).

TABLE V

EFFECT OF NUCLEOSIDE TRIPHOSPHATES ON GTPase AND ADENYLATE CYCLASE ACTIVITIES

ITP and UTP were purified from GTP contaminations as described in Materials and Methods. CTP was used without purification. GTPase was assayed as described under Materials and Methods using a membrane concentration of 0.20 mg/ml. In the adenylate cyclase reaction the ATP concentration was 10 μM, and the membranes concentration was 0.25 mg/ml. The catecholamine used was isoproterenol, 50 μM.

Nucleotide addition (0.1 mM)	GTPase activity (pmol $^{32}P_i$/mg per min)		Catecholamine stimulated adenylate cyclase activity	
	"Basal"	Catecholamine-stimulated	pmol/cyclic AMP/mg per min	% increase caused by the nucleotide
No addition	6.3	5.4	3.2	—
CTP	7.1	5.7	3.55	11
UTP	4.6	4.4	3.95	23
ITP	1.5	0.7	7.35	130
GTP	—	—	7.2	125

TABLE VI

EFFECTS OF SH REAGENTS, PHOSPHOLIPASE C AND LUBROL PX ON GTPase AND ADENYLATE CYCLASE ACTIVITIES

The concentration of membranes used in the GTPase assays was 0.2—0.3 mg/ml and in the adenylate cyclase assays 0.5—0.8 mg/ml. Isoproterenol was 50 μM and NaF was 10 mM. Experiment I. Membranes (0.6 mg/ml) in 10 mM Tris · HCl (pH 8.1) and 0.1 mM EDTA were treated with MalNEt for 30 min at 0°C. Incubation was terminated by the addition of β-mercaptoethanol, 5 mM in excess of [MalNEt]. Experiment II. Membranes (0.5 mg/ml) in 10 mM Tris · HCl (pH 7.9) and 0.1 mM EDTA were treated for 15 min at 30°C with 0.1 mM I *p*-chloromercuribenzoic acid, cooled and then washed with imidazole buffer. Membranes (1.4 mg/ml) in 10 mM imidazole · HCl (pH 6.7) and 1 mM β-mercaptoethanol were incubated for 15 min at 30°C with or without phospholipase C from B. cereus, 0.1 mg/ml. Experiment III. Membranes (1.6 mg/ml) were incubated for 10 min at 23°C in the presence of 50 mM imidazole · HCl (pH 6.7) and 1 mM β-mercaptoethanol, with or without 1 mg/ml of lubrol PX.

Pretreatment	GTPase activity (pmol $^{32}P_i$/mg per min)		Adenylate cyclase activity (pmol cyclic AMP/mg per min)	
	Basal	Catecholamine-stimulated	Isoproterenol	NaF
Experiment I				
No addition	10.8	4.5	89	233
0.5 mM MalNEt at 0°C	7.0	5.1	11	30
10 mM MalNEt at 0°C	2.8	4.6	0.6	4.3
Experiment II				
No addition	7.1	5.1	48	243
0.1 mM *p*-chloromercuribenzoic acid at 30°C	1.1	0.4	0	0
Phospholipase C	3.7	0.2	2.0	208
Experiment III				
No addition	11.3	6.4	178	275
Lubrol PX	4.6	0.5	13.5	183

It was found that Mg^{2+} was required for he catecholamine-stimulated GTPase reaction and the Mn^{2+} could efficiently substitute for Mg^{2+}. Since Ca^{2+} and Sr^{2+} greatly increased the non-specific hydrolysis of GTP, it was difficult to assess the effects of these cations on specific hydrolysis.

Treatment of the membrane with *p*-chloromercuribenzoic acid which is known to inactivate the adenylate cyclase caused complete inhibition of both the basal and the catecholamine-stimulated GTPases (Table VI). On the other hand, MalNEt was found to act more selectively. As shown in Table VI, the order of sensitivity to inactivation by MalNEt in the cold is adenylate cyclase > basal GTPase >> catecholamine-stimulated GTPase. Treatment with 10 mM MalNEt at 0°C resulted in 98% inhibition of the adenylate cyclase, 75% inhibition of the basal GTPase, and in little, if any, effect on the catecholamine-stimulated GTPase. This finding suggests that the GTPase reaction is not dependent on the catalytic production of cyclic AMP by the adenylate cyclase. At 30°C the catecholamine-stimulated GTPase became sensitive to MalNEt although it was inactivated to a lesser extent than the basal GTPase. At this latter temperature isoproterenol selectively increased the sensitivity of the catecholamine-stimulated GTPase to MalNEt, so that the inactivation curves of the two GTPases almost coincided (Fig. 5).

Pretreatment of the membranes with either phospholipase C or lubrol PX,

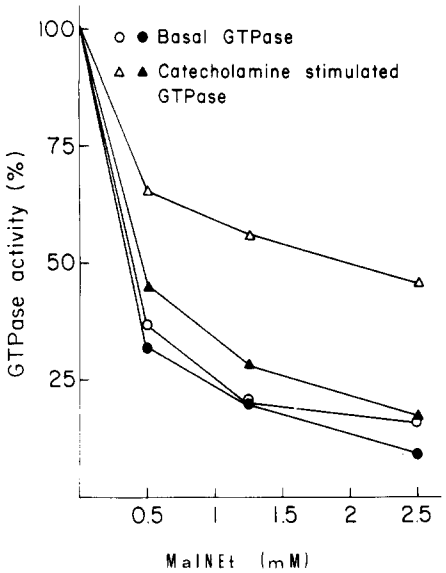


Fig. 5. Inhibition of GTPase activity by MalNEt in the presence and absence of isoproterenol. Membranes, 0.65 mg/ml in a medium containing 10 mM Tris · HCl buffer (pH 7.9), 0.1 mM EDTA and 0.5 mM ascorbate were incubated for 10 min at 30°C with the indicated concentrations of MalNEt in the absence (○,△) and presence (●,▲) of 50 μM isoproterenol. Alkylation was terminated by the addition of β-mercaptoethanol to give a 5 mM concentration in excess of MalNEt. To determine the "basal" GTPase in systems preincubated with isoproterenol, propranolol (50 μM) was added. The catecholamine-stimulated GTPase was determined using 50 μM isoproterenol. Membranes concentration was 0.32 mg/ml.

almost completely abolished the catecholamine-stimulated adenylate cyclase activity while the ability of fluoride to stimulate the enzyme was retained. These treatments also caused almost complete loss of the catecholamine-stimulated GTPase activity and 50—60% inhibition of the "basal" GTPase activity. In contrast to the partially purified membranes intact erythrocytes did not show specific GTPase activity. Furthermore, incubation of whole erythrocytes with trypsin or chymotrypsin prior to the preparation of erythrocyte membranes had no effect on the "basal" and hormone-stimulated GTPase activities. On the other hand, treatment of the membranes with the proteolytic enzymes resulted in a complete inactivation of both GTPases (data not shown). These results indicate that the GTPases are localized on the inner surface of the erythrocyte membrane. In the present study it was confirmed that proteolysis of whole turkey erythrocytes does not affect the stimulation of the adenylate cyclase by catecholamine (see also ref. 23).

Discussion

Choice of conditions for selective assay of specific GTPase activity

The GTPase assay which has been described was designed to measure a minimal GTPase activity in the membrane, despite the presence of highly active nucleoside triphosphatases. This required an assay detecting the full extent of only those GTPase activities having a high affinity for GTP ($K_m \approx 0.1$ μM) and effectively discriminating between adenine and guanine nucleotides. These

properties are expected for a GTPase activity in the adenylate cyclase system [4]. It should also be noted that App(NH)p is a substrate for the adenylate cyclase [1] and the other constituents of the GTPase assay have been used for the determination of the adenylate cyclase activity.

Coupling of GTPase activity to the β-adrenergic receptor

The stimulation of GTP hydrolysis by catecholamines clearly indicates that the turkey eryghrocyte membrane contains a GTPase which is coupled to the β-adrenergic receptor: the specificity of the β-adrenergic receptor and its affinity for isoproterenol are very similar when determined either by the GTPase assay, or by the adenylate cyclase reaction (Tables III, IV and Fig. 3). Furthermore, treatments with lubrol PX or phospholipase C which uncouple the β-adrenergic receptor from the adenylate cyclase abolished the stimulation of GTP hydrolysis by catecholamines (Table VI). Two lines of evidence demonstrate the stimulation of the GTPase by catecholamines is not an indirect effect mediated through cyclic AMP:

(1) The cyclic nucleotide could not substitute for the hormone as a stimulator of GTPase activity (Table IV).

(2) The stimulation of GTP hydrolysis was found to be independent of the catalytic activity of the adenylate cyclase since the latter could be selectively inhibited by treatment by MalNEt at 0°C (Table VI). In this respect, the catecholamine stimulated GTP hydrolysis resembles the permantent activation of the adenylate cyclase by Gpp(NH)p and isoproterenol which takes place in the absence of a catalytic production of cyclic AMP [6,7,9].

The relationship between the basal and the catecholamine-stimulated GTPase activities

The similar specificity (Table V) and the high affinity for GTP of both the basal and the catecholamine stimulated GTPase ($K_m \approx 0.1$ μM) raises the question as to whether the two GTPase activities are due to one enzyme which acts more efficiently in the presence of catecholamines. The fact that at 0°C MalNEt preferentially inactivated the basal GTPase while it had only littly effect on the catecholamine-stimulated GTPase argues against this hypothesis (Table VI). The increased inactivation of the catecholamine stimulated GTPase by MalNEt in the presence of isoproterenol (Fig. 5) may be interpreted as follows. Both the "basal" and the catecholamine stimulated GTPases have a common catalytic component which exists in the membrane in two different states. In one state this component is coupled to the β-adrenergic receptor and has an "inhibited" conformation (catecholamine-stimulated GTPase) whilst in the other state the same component is not coupled to the receptor and is permanently active (basal GTPase). Upon activation by catecholamines, the "inhibited" GTPase gains the active conformation of the basal GTPase and in this condition the two GTPases have the same sensitivity to MalNEt (Fig. 5).

The role of GTPase activity in the adenylate cyclase system

Since GTP activates and GDP inhibits adenylate cyclase activity [10], the finding that catecholamines cause an increased hydrolysis of GTP as well as activation of the adenylate cyclase, deserves a comment. It should be noted,

that the hydrolysis of GTP by the catecholamine-stimulated GTPase, is 10—30-fold slower than the adenylate cyclase reaction. We suggest that the catecholamines activated state of the adenylate cyclase has an increased ability to hydrolyze GTP. Nevertheless, the hydrolysis is slow enough to allow for an almost continuous presence of GTP at the regulatory site. Hydrolysis of GTP is required ultimately in order to allow the system to return to the inactive state. The present findings argue against the suggestion that hormone stimulation of the adenylate cyclase is mediated by an inhibition of rapid GTP hydrolysis.

In contrast to catecholamines, fluoride ions caused a small inhibition of the GTPase activity. This might be due to a different mechanism by which catecholamines and fluoride activate the adenylate cyclase. Indeed no requirement was observed for GTP in stimulation of the adenylate cyclase by fluoride. Moreover addition of GTP inhibited the fluoride-stimulated adenylate cyclase activity of fat cell membranes [1].

Preliminary experiments with the hepatic membrane adenylate cyclase system showed a "basal" GTPase of specific activity 10 times higher than that of turkey erythrocyte membrane and a 50-fold higher non-specific NTPase activity. No stimulation of GTP hydrolysis by glucagon and epinephrine was observed. Stimulation of the GTPase in the hepatic system to an extent stimilar to that found in the turkey erythrocyte membrane, however would have only resulted in an increase of 3—5% of the total GTP hydrolysis in the assay system. In pigeon erythrocyte membrane isoproterenol caused a 20% increase in GTP hydrolysis. Apparently the high activities of NTPases and basal GTPase make it difficult to demonstrate a hormone-stimulated GTPase in membranes other than the erythrocyte membrane. Nevertheless, the difference between GTP and its metabolically stable analogues as activators of a wide range of hormone-stimulated adenylate cyclases makes it likely that a GTPase plays a general role in adenylate cyclase systems and is not confined to the turkey erythrocyte membrane.

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