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Prostaglandin-stimulated GTP hydrolysis associated with activation of adenylate cyclase in human platelet membranes

(cholera toxin/*N*-ethylmaleimide/epinephrine)

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ABSTRACT **In membranes purified from human blood platelets, basal guanosine triphosphate (GTP) hydrolysis is reduced by a factor of ≈6 by exposure to *N*-ethylmaleimide (10 mM). This decreased background enables the detection of an additional GTP hydrolysis in the presence of prostaglandin E_1 (PGE_1). The PGE_1-stimulated GTPase has several properties correlated with PGE_1-stimulated adenylate cyclase in this preparation. The two enzymes have similar dose–response relationships (half-maximal stimulation at 0.1 μM PGE_1). Exposure to cholera toxin blocks the PGE_1-stimulated GTPase and activates adenylate cyclase. Both enzymes are activated by submicromolar concentrations of GTP, although the K_m for the GTPase is about 10 times greater than that for the adenylate cyclase. The data are discussed in relation to the hypothesis that hormone-stimulated adenylate cyclase (*i*) is activated as a regulatory component binds a molecule of GTP and (*ii*) is deactivated as this molecule is hydrolyzed.**

Guanyl nucleotides play a central role in the hormonal stimulation of adenylate cyclase in various tissues. For cases where the point has been carefully studied, such stimulation is absolutely dependent on the presence of GTP or certain of its analogs (1). The hypothesis has arisen that a regulatory subunit of the cyclase (*i*) binds GTP as the cyclase is activated and (*ii*) hydrolyzes the GTP as the cyclase is again deactivated. This concept has received strongest support for the turkey erythrocyte membrane. Because this preparation has a low basal level of GTP hydrolysis, it was possible to observe an increment of GTPase activity produced by adrenergic hormones (2, 3). This hormone-stimulated GTPase has several properties expected of a system that regulates the adenylate cyclase activity of these membranes.

For other membrane preparations, there is less direct evidence that GTP is hydrolyzed by a component of adenylate cyclase. A persistent activation is produced by GTP analogs whose β,γ-phosphate bond cannot be hydrolyzed enzymatically and by cholera toxin, which blocks the GTPase in turkey erythrocytes (4). More direct proof would involve a demonstration of GTP hydrolysis that shares the properties of hormone-stimulated adenylate cyclase: (*i*) the dose–response relationship should resemble that for cyclase stimulation; (*ii*) the GTPase should be active at low GTP concentration, with a K_m of $<1 \times 10^{-6}$ M; and (*iii*) the GTPase should be blocked by cholera toxin. We have verified these concepts for the prostaglandin-stimulated adenylate cyclase of membranes purified from human platelets. The measurements are made possible by the fact that exposure to *N*-ethylmaleimide (MalNEt) markedly decreases basal GTP hydrolysis but apparently not hormone-stimulated GTPase associated with cyclase (2).

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Adenylate cyclase in human platelet membranes displays the additional property of inhibition by α_2-adrenergic agonists (5). This inhibition requires guanyl nucleotides, albeit at a higher concentration than required for adenylate cyclase activation (6, 7). The experiments thus afforded the opportunity to test whether this inhibition is accompanied by a change in GTPase activity.

MATERIALS AND METHODS

ATP (GTP-free) and guanyl-5′-yl imidodiphosphate (p[NH]ppG) were from Boehringer Mannheim; prostaglandin E_1 (PGE_1) was a gift from Upjohn; adenyl-5′-yl imidodiphosphate (p[NH]ppA) was from ICN or Sigma; 2-mercaptoethanol was from Eastman; radiochemicals were from The Radiochemical Centre, Amersham. Cholera toxin was obtained directly from Makor Chemicals, Jerusalem, or through its distributor, Sigma. Other chemicals were from Sigma.

Platelet Membrane Preparation. Whole blood was obtained from donors who had taken no aspirin-containing drugs for 2 wk prior to blood donation, and platelet membranes were prepared by the glycerol-lysis technique (8) as described by Steer and Wood (7). At all stages of the experiments, pelleted membranes were resuspended gradually by using a Teflon rod (7). Adenylate cyclase and GTPase assays were performed within 6 hr of blood donation. Protein was determined by the method of Lowry *et al.* (9) with bovine serum albumin as a standard.

Turkey Erythrocyte Membranes. These were prepared as described by Steer and Levitzki (10).

Cholera Toxin Treatment. This was performed essentially as described by Kaslow *et al.* (11). Cholera toxin (200 μg/ml) was activated by incubation for 10 min at 30°C in 2 mM dithiothreitol/50 mM sodium phosphate buffer, pH 7.1/100 mM NaCl/5 mM $MgCl_2$, with 0.2 mg of ovalbumin per ml. This solution was added in equal volume to membranes suspended (1.5 mg/ml) in 1 mM ATP/1 mM nicotinamide adenine dinucleotide/200 μM GTP/50 mM sodium phosphate buffer, pH 7.1, with 100 units of creatine kinase per ml. The toxin and membranes were incubated for 10 min at 30°C, and the reaction was stopped by spinning at 20,000 × *g* for 10 min. Membranes were then washed twice in 10 mM Tris·HCl, pH 8/0.1 mM EDTA/0.25 M sucrose, followed by one wash in Tris/EDTA without sucrose. Pellets were resuspended in 10 mM Tris. This suspension was divided into portions destined for the adenylate

Abbreviations: MalNEt, *N*-ethylmaleimide; PGE_1, prostaglandin E_1; p[NH]ppA, adenyl-5′-yl imidodiphosphate; p[NH]ppG, guanyl-5′-yl imidodiphosphate.

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cyclase assay (see below) or for MalNEt treatment followed by the GTPase assay.

Adenylate Cyclase Assay. The reaction mixture had a final volume of 0.2 ml containing 0.1–0.3 mg of platelet membrane protein per ml. The enzyme was assayed under "standard" and "GTPase" conditions. In the former case, the concentrations of agents in the assay were: Tris·HCl (pH 8.0), 30 mM; $MgCl_2$, 10 mM; caffeine, 33 mM; creatine phosphate, 5 mM; creatine kinase, 50 units/ml; and [α-^{32}P]ATP (50–100 cpm/pmol), 0.2 mM. Incubations were for 10 min at 30°C. Under "GTPase" assay conditions, the incubation was for 5 min at 37°C, and final concentrations of reagents in the assay mixture were exactly as described for the GTPase assay (see below), except that the radiochemical was [α-^{32}P]ATP.

In either case, the reaction was terminated by addition of a solution containing 4 mM ATP, 1.4 mM cAMP, and 2% sodium dodecyl sulfate. [^{3}H]cAMP (5–10,000 cpm per assay tube) was added, and [^{32}P]cAMP was collected by the method of Salomon *et al.* (12). Recovery of [^{32}P]cAMP was corrected by using the value obtained for [^{3}H]cAMP recovery (50–80%).

MalNEt Treatment. This was performed essentially as described by Cassel and Selinger (2). Membranes (0.5–1 mg of protein per ml) were incubated in 10 mM MalNEt for 30 min at 0°C. The incubation was terminated by addition of 15 mM 2-mercaptoethanol. After an additional 5 min at 0°C, the reaction was diluted 1:10 (vol/vol) with 50 mM imidazole·HCl, and membranes were pelleted at 20,000 × *g* for 10 min. Membranes were resuspended in imidazole buffer and used for the GTPase assay.

GTPase Assay. This was performed by liberation of $^{32}P_i$ from [γ-^{32}P]GTP as described by Cassel and Selinger (13). The reaction mixture (final volume, 100 μl) contained 0.25 μM [α-^{32}P]GTP (2.5–10 × 10^3 cpm/pmol), 0.1 mM ATP, 6 mM $MgCl_2$, 0.5 mM p[NH]ppA, 2 mM 2-mercaptoethanol, 3 mM creatine phosphate, 0.1 mM EGTA, 3 units of creatine kinase, and 50 mM imidazole·HCl buffer (pH 6.7). The reaction tubes, minus the membranes, were preincubated at 37°C for 15 sec. The reaction was initiated by adding 10–40 μg of the membranes and was terminated after 10 min by adding 0.5 ml of a suspension of 5% charcoal (acid washed, Norit A) in ice-cold 20 mM phosphate buffer (pH 7.1). After at least 5 min at 30°C, the suspension was centrifuged (1000 × *g* for 5 min) and 200 μl of the supernatant was assayed using Cerenkov radiation. With this method, 1–5% of the added $^{32}P_i$ was recovered in assays without added membranes, presumably because the [γ-^{32}P]GTP hydrolyzed spontaneously during storage; this value was subtracted from the data with membranes. Assays were performed in triplicate or quadruplicate.

RESULTS

Critique of the MalNEt Method. With the GTPase assay system developed by Cassel and Selinger (2, 13), human platelet membranes had a basal activity of about 60 pmol/min per mg of protein (Table 1). PGE_1 produced a barely significant increase of about 5 pmol/min per mg. The basal activity was reduced by a factor of ≈6 by exposure to MalNEt at 10 mM, rendering the PGE_1 stimulation more evident. In eight preparations studied after MalNEt exposure, the PGE_1 stimulation amounted to an increase of 46 ± 7% (mean ± SEM) over basal activity. A higher MalNEt concentration reduced both the basal and PGE_1-stimulated activity, producing no further improvement in the resolution of the assay (Table 1).

The MalNEt treatment has the disadvantage of blocking adenylate cyclase activity. Exposure to MalNEt might also modify the prostaglandin-stimulated GTPase activity, vitiating studies with this technique. To evaluate this possibility, we exploited the turkey erythrocyte system, whose low basal GTPase activity allows measurements of epinephrine stimulation both before and after MalNEt treatment. The data (Fig. 1) show that MalNEt treatment produced little or no change in the K_m for GTP and a small (44%) decrement in the maximal GTPase activity. Thus, hormone-stimulated GTPase is not greatly affected by the MalNEt treatment.

Table 1. Effects of MalNEt treatment on GTPase in human platelet membranes

MalNEt, mM	GTPase activity,* No additions	With epinephrine	With PGE_1	With PGE_1 and epinephrine
0	59.3 ± 1.0	62.9 ± 1.8	64.5 ± 1.6	65.7 ± 1.9
10	9.7 ± 0.4	10.2 ± 0.2	14.8 ± 0.4	14.8 ± 0.6
50	6.7 ± 0.2	6.7 ± 0.3	9.8 ± 0.5	9.6 ± 0.4

Membranes were exposed to MalNEt for 30 min at 0°C. Epinephrine was 100 μM with 1 μM propranolol.
* In pmol/min per mg of protein, expressed as mean ± SEM of four replicates.

Dose–Response Characteristics for the GTPase and Cyclase. Earlier studies have shown that prostaglandin-stimulated adenylate cyclase is stimulated to half-maximal activity at PGE_1 concentrations on the order of 0.1 μM (7). The present experiments confirmed these data and showed that the PGE_1-stimulated GTPase displays strikingly similar dose–response characteristics (Fig. 2).

Effects of Cholera Toxin. Cholera toxin affects human platelet membranes analogously to its action on turkey erythrocytes: it activates adenylate cyclase (14), presumably by blocking hormone-stimulated GTPase (Table 2). Because human platelet membranes have significant basal adenylate cyclase, it was of interest to determine whether basal GTPase activity was also affected by exposure to cholera toxin. There was a small decrease in the experiment of Table 2, but this was not observed

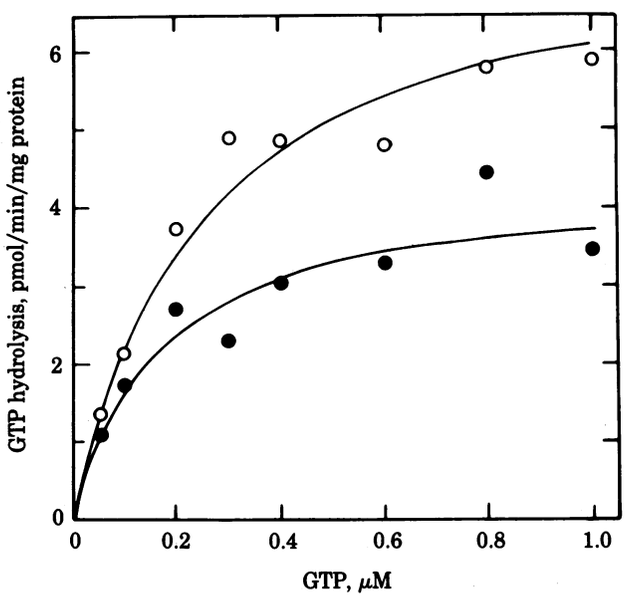

FIG. 1. Effects of MalNEt treatment on epinephrine-stimulated GTPase in turkey erythrocyte membranes. Data show the increase in GTP hydrolysis in the presence of 0.1 mM l-epinephrine. Lines are theoretical hyperbolic functions. ○, Normal (V_{max}, 7.7 pmol/min per mg of protein; K_m, 2.5 × 10^{-7} M). ●, MalNEt-treated (V_{max}, 4.4 pmol/min per mg of protein; K_m, 1.7 × 10^{-7} M).

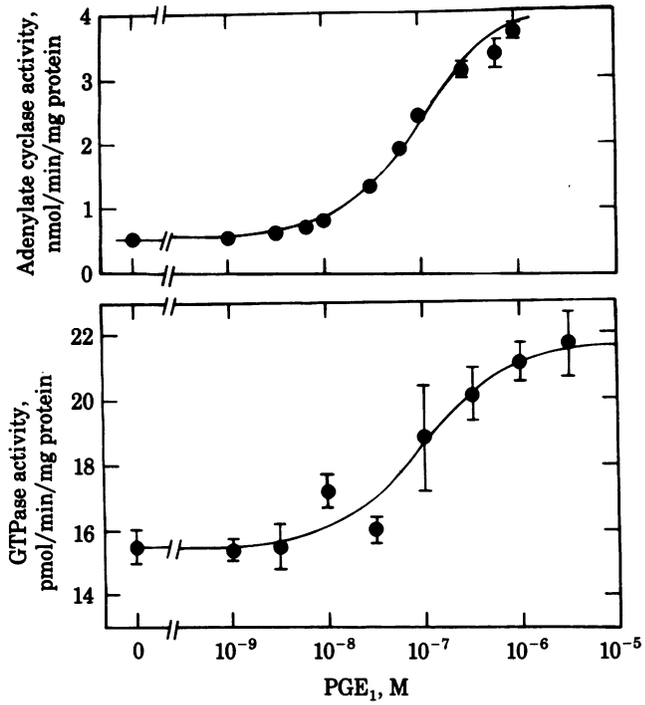

FIG. 2. Dose–response characteristics for the PGE_1-stimulated adenylate cyclase (*Upper*) and GTPase (*Lower*). Lines are theoretical hyperbolic functions. (*Upper*) V_{max}, 3.5 nmol/min per mg of protein; K_m, 1×10^{-7} M. (*Lower*) V_{max}, 6.2 pmol/min per mg of protein; K_m, 1×10^{-7} M. Standard errors of the mean are shown where they exceed the size of the symbols.

in a second experiment under similar conditions. In both experiments, hormone-induced GTPase was reduced by a factor of ≈3 after exposure to cholera toxin. Therefore, the cholera toxin specifically decreases hormone-stimulated GTPase, as observed for turkey erythrocytes (4).

GTP Concentration Dependence. Human platelet membranes showed both basal and PGE_1-stimulated GTPase for GTP concentrations in the range of 0.05–10 μM (Fig. 2). In an experiment to explore the range between zero and 2 μM, we found that the basal GTPase was characterized by a component with a K_m of $\approx 1 \times 10^{-7}$ M and a second component, presumably with a much higher K_m, whose rate increased linearly with GTP concentration (data not shown).

However, the prostaglandin-stimulated GTPase was characterized by a K_m several times larger than 1×10^{-7} M. In the experiments of Fig. 3 and Fig. 4*b*, the K_m was 6×10^{-7} M; and two similar experiments gave values of 4 and 6×10^{-7} M. By contrast, the prostaglandin-stimulated adenylate cyclase is activated by much lower GTP concentrations (6, 7).

Table 2. Effects of cholera toxin on GTPase and adenylate cyclase

Addition	Enzyme activity*	
	No pretreatment	Cholera toxin
	GTPase	
None	11.5 ± 0.9	7.5 ± 0.7
PGE_1	19.9 ± 1.1	10.3 ± 0.7
	Adenylate cyclase	
None	50 ± 2.5	180 ± 9
GTP	100 ± 5	690 ± 35
p[NH]ppG	507 ± 25	491 ± 25

* In pmol/min per mg of protein, expressed as mean ± SD.

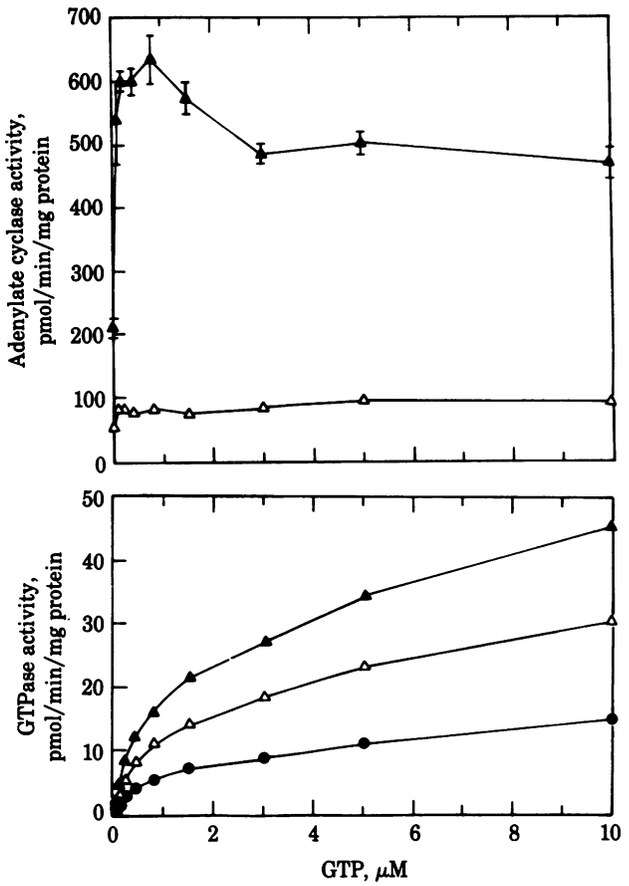

FIG. 3. GTP concentration dependence of the adenylate cyclase (*Upper*) and GTPase (*Lower*). ▲, PGE_1; △, basal; ●, PGE_1-stimulated GTPase. These data are analyzed further in Fig. 4*B*.

To provide an accurate comparison between the two enzymes, we assayed adenylate cyclase under the same conditions used in the GTPase assay, although the MalNEt treatment was omitted. (The adenylate cyclase activity was only about 1/6th of the normal value under these suboptimal conditions.) Half-maximal stimulation was observed at 0.04 μM GTP. There was some activity even with no added GTP, perhaps because the membrane preparation itself contributed guanyl nucleotides. If so, the K_m for adenylate cyclase was about 6×10^{-8} M, or about 1/10th the K_m for GTP hydrolysis.

If the membrane preparation contributed GTP, this would also artifactually increase the measured K_m for the GTPase by the amount contributed. Two observations render it unlikely that such a mechanism significantly distorted the GTPase results. First, a K_m of 10^{-7} M was measured for basal GTPase, suggesting that endogenous GTP is below this level. Second, control experiments showed that the rate of $^{32}P_i$ release was linear with the amount of membrane protein added.

Effects of Epinephrine. We studied the effects of epinephrine on GTPase and on adenylate cyclase at two GTP concentrations, 0.25 μM (the usual value in our experiments) and 5 μM (where the largest epinephrine inhibition of adenylate cyclase is observed). At both concentrations, epinephrine produced clearcut inhibition of adenylate cyclase, and PGE_1 produced clearcut stimulation of GTPase; yet epinephrine had no effect on GTPase in either the presence or absence of PGE_1 (Table 3). The contrast is particularly clear at 5 μM GTP in the absence of PGE_1: epinephrine decreased adenylate cyclase activity to 56% of the control value, with no measurable change in GTPase activity.

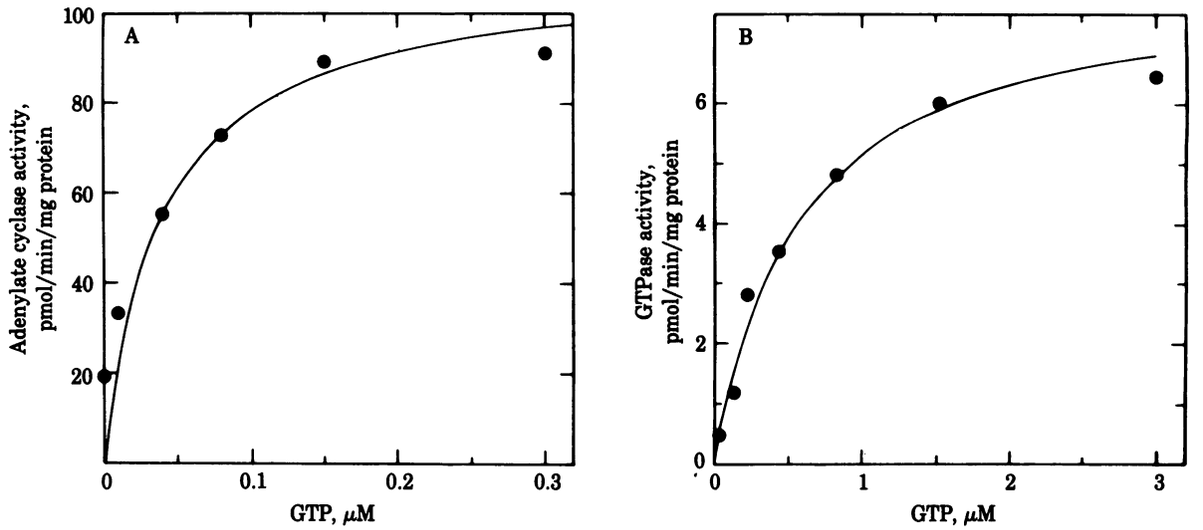

FIG. 4. Further comparison of GTP concentration effects on PGE_1-stimulated adenylate cyclase (*A*) and GTPase (*B*). The adenylate cyclase activity was measured under the same conditions as those used in the GTPase assay. For *B*, a straight line was fit to the PGE_1-stimulated GTPase at GTP concentrations ≥ 3 μM in the experiment of Fig. 3. This line presumably represents a low-affinity PGE_1-stimulated GTPase and was subtracted from the data. This correction is equivalent to subtracting the radioactivity released at high concentrations of unlabeled GTP in the presence of PGE_1 (2). Resulting values are plotted. Solid lines are theoretical hyperbolic functions. (*A*) V_{max}, 110 pmol/min per mg of protein; K_m, 4×10^{-8} M. (*B*) V_{max}, 8.2 pmol/min per mg of protein; K_m, 6×10^{-7} M.

DISCUSSION

GTPase and Cyclase. The data leave little doubt that the prostaglandin-stimulated GTPase activity is closely correlated with activation of adenylate cyclase. The dose–response characteristics match for the two enzymes, they are affected as expected by cholera toxin, and both are activated by GTP at submicromolar concentrations. This correlation seems more direct than for the prostaglandin-stimulated GTP hydrolysis recently reported for human mononuclear cells (15). Good correlations between GTPase and adenylate cyclase also have been reported for activation by pancreozymin (16) and glucagon (17) in addition to the original observations with β-adrenergic agonists in erythrocytes (2, 18).

There is, however, an interesting discrepancy between the detailed GTP concentration dependence of the two enzymes. Adenylate cyclase activity is half-maximal at about 4×10^{-8} M GTP; the K_m for the GTPase is 10-fold higher. The K_m values for the two enzymes would be equal in the simple scheme where (*i*) a molecule of cyclase is activated as a molecule of GTP binds to a regulatory component and (*ii*) cyclase is deactivated as GDP and P_i are released. Evidently events at the GTP binding site are coupled in a less direct fashion to the cyclase.

Table 3. Comparison of epinephrine effects on GTPase and adenylate cyclase

GTP, μM	% change by epinephrine	
	GTPase	Adenylate cyclase
	Without PGE_1	
0.25	+2 ± 4	−12 ± 3
5.00	+7 ± 8	−44 ± 10
	With PGE_1, 1.0 μM	
0.25	−9 ± 8	−10 ± 6*
5.00	−5 ± 13	−26 ± 5

Epinephrine concentration was 10 or 100 μM, with 1 μM propranolol. Values are expressed as the mean ± SEM of four to six experiments.
* Single experiment.

Inhibition by Epinephrine. Our experiments disclose no effect of epinephrine on GTPase under conditions where epinephrine produces clear inhibition of adenylate cyclase. These data must be viewed as incomplete because (*i*) the GTPase measurements have a poor signal-to-noise ratio in the absence of MalNEt treatment (Table 1); (*ii*) MalNEt treatment, which reveals the PGE_1-stimulated GTPase, might conceivably have modified some component of the inhibitory system. Nonetheless, the data are consistent with the observation that epinephrine does not alter the dynamics of cyclase activation and inactivation by GTP analogs; these dynamics are thought to provide a measure of the rate constants for the GTPase cycle (unpublished observations).

Note Added in Proof. Aktories and Jacobs (19) have reported that epinephrine stimulates a GTPase in human platelet membranes.

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