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SYNERGISTIC INHIBITION OF HUMAN PLATELET ADENYLATE CYCLASE BY STABLE GTP ANALOGS AND EPINEPHRINE

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Adenylate cyclase inhibition by stable GTP analogs and their interaction with epinephrine were studied in human platelet membranes. Whereas basal enzyme activity was increased by these nucleotides, the stable GTP analogs decreased the adenylate cyclase activity stimulated by fluoride or forskolin by maximally 60 to 70%, with the potency order, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) > guanyl-5′-ylimidodiphosphate > guanyl-5′-ylmethylenediphosphate. The inhibition of the forskolin-stimulated enzyme by GTPγS was half-maximal at about 4 nM, occurred after a time lag period, which was inversely related to the GTPγS concentration, and was resistant to washing of the membranes. Prostaglandin E_1-stimulated activity exhibited a biphasic response towards GTPγS, with activation occurring at low (1 nM) and inhibition at higher GTPγS concentrations. The inhibitory effect of GTPγS was competitively antagonized by GTP. This antagonism was prevented by epinephrine, which inhibited the stimulated platelet adenylate cyclase in the presence of GTP to the same degree as observed with GTPγS alone. In the absence of GTP, epinephrine largely diminished the time lag required for the inhibitory action of GTPγS. Furthermore, the decrease in final activity induced by GTPγS was amplified by epinephrine. Whereas the acceleration of the inhibitory action of GTPγS was observed at low and high GTPγS concentrations, the amplification by epinephrine was observed only at submaximally effective concentrations of GTPγS.

Introduction

The hormone-sensitive adenylate cyclase (ATP pyrophosphate lyase (cyclizing);EC 4.6.1.1) appears to be regulated by two distinct guanine nucleotide sites, i.e., N_s and N_i, mediating enzyme stimulation and inhibition, respectively [1]. Stimulation of adenylate cyclase via N_s is apparently initiated by the binding of GTP to N_s following hormone-receptor interaction (Ref. 2, see Ref. 3 for a review). N_s can also be activated by stable GTP analogs. This process is time-dependent and can be accelerated and amplified by stimulatory hormones. Due to their resistance to the inactivating GTPase [4], the stable GTP analogs can cause a persistent activation of N_s and thereby a persistent activation of the adenylate cyclase. However, stable GTP analogs not only stimulate adenylate cyclase. As shown in various cell types, these nucleotides can also cause adenylate cyclase inhibition [5–15]. In some systems, the inhibition was observed with the basal, unstimulated enzyme [5,6,13,14], whereas in most cellular systems stable GTP analogs only inhibited the enzyme stimulated by other agents, such as cholera toxin, prostaglandin E_1 or forskolin. The stable GTP analog-

Abbreviations used: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Gpp[NH]p, guanyl-5′-ylimidodiphosphate; Gpp[CH_2]p, guanyl-5′-ylmethylenediphosphate; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate).

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induced inhibition was resistant to washing of the membranes [6,7,12,15] and it was obliterated by pretreatment of the membranes with *N*-ethylmaleimide or trypsin [13,15] and in the presence of Mn^{2+} (at least 1 mM) [12,15]. These treatments can also abolish hormone-induced adenylate cyclase inhibition [16–20]. Finally, adenylate cyclase inhibition by stable GTP analogs was also observed in cyc^- variants of S49 lymphoma cells, which are deficient in N_s [14,15]. Therefore, it has been suggested that stable GTP analogs inhibit adenylate cyclase by interaction with the inhibitory guanine nucleotide site, N_i, and that the mechanisms of activation and inactivation of N_i are similar to those reported for N_s [12–15].

If the activation mechanisms of N_i are similar to those of N_s, the inhibitory action of stable GTP analogs should be accelerated and amplified by an inhibitory hormone. Human platelet adenylate cyclase can be inhibited by epinephrine acting via α_2-adrenoceptors [21,22]. This hormone-induced inhibition is a GTP-dependent process [23], suggesting that the inhibition involves the inhibitory nucleotide site, N_i. As shown before, the platelet enzyme can also be inhibited by the stable GTP analog, guanyl-5′-ylimidodiphosphate (Gpp[NH]p) [7,11]. Therefore, we studied the characteristics of adenylate cyclase inhibition by stable GTP analogs in human platelet membranes and particularly the question whether and how epinephrine interferes with this guanine nucleotide-induced inhibition. Here we report that stable GTP analogs can cause a time-dependent and persistent inhibition of the human platelet adenylate cyclase and that epinephrine can accelerate and amplify the inhibitory effect of these analogs.

Materials and Methods

Materials. ATP, GTP, Gpp[NH]p, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), guanyl-5′-ylmethylenediphosphate (Gpp[CH_2]p), guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) and creatine kinase were obtained from Boehringer Mannheim. Prostaglandin E_1, epinephrine and creatine phosphate were from Sigma. Forskolin was kindly donated by Dr. H. Metzger, Hoechst AG, Frankfurt. [α-^{32}P]ATP was prepared enzymatically [24]. All other materials were from previously described sources [15,16].

Preparation of human platelet membranes. Crude membranes of human platelets were prepared as described [16] with 5 mM EDTA present during the preparation procedure.

Adenylate cyclase assay. Adenylate cyclase activity was determined, if not otherwise indicated, in a reaction mixture containing 50 μM ATP, 2 mM $MgCl_2$, 0.1 mM ethylene glycol bis(β-aminoethylether) *N*, *N*′-tetraacetic acid, 0.1 mM cyclic AMP, 1 mM 3-isobutyl-1-methylxanthine, 1 mM dithiothreitol, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase, 2 mg/ml bovine serum albumin and 50 mM triethanolamine-HCl, pH 7.4, in a final volume of 100 μl. Reactions were initiated by the addition of platelet membrane preparation (10–20 μg protein/tube) to the reaction mixture and continued for 15 min at 25°C. Thereafter, [α-^{32}P]ATP (0.2–0.4 μCi/tube) was added and the reactions were continued for 10 min at 25°C. In the time-course experiments shown, reactions were initiated by the addition of platelet membranes to the reaction mixture. After an incubation period of 7 min at 25°C, [α-^{32}P]ATP was added without and with the guanine nucleotides at the indicated concentrations. At various time points, in 100-μl aliquots the cyclic [^{32}P]AMP formed was determined. Incubations were terminated and cyclic AMP was isolated as described [21]. Protein was determined according to Lowry et al. [25] with human serum albumin as standard. All experiments were performed in triplicate with an intraassay variation of less than 5% of the means, and were repeated at least twice.

Results

Under the assay conditions used, the stable GTP analog, GTPγS, increased basal platelet adenylate cyclase activity 2-fold, with a half-maximal and maximal effect at 10 and 1000 nM GTPγS, respectively (Fig. 1). However, in the presence of NaF (10 mM) and forskolin (20 μM), which increased the activity about 10- and 35-fold, respectively, GTPγS caused a concentration-dependent inhibition, which was half-maximal at 4 and 10 nM GTPγS, respectively. GTPγS exhibited a biphasic effect on the adenylate cyclase activity stimulated by the hormonal factor, prostaglandin E_1 (10 μM). At 1 nM GTPγS, the activation of the

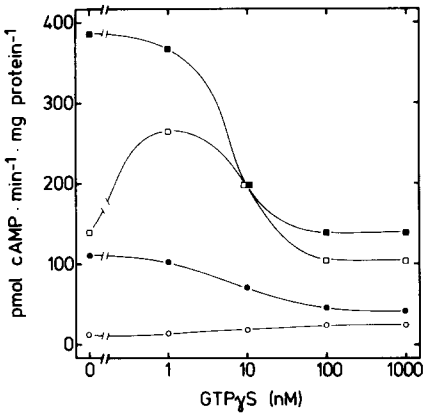

Fig. 1. Influence of GTPγS on basal and stimulated forms of human platelet adenylate cyclase. Adenylate cyclase activity was determined in human platelet membranes in the absence (○———○) and presence of NaF (10 mM) (●———●), prostaglandin E_1 (10 μM) (□———□) or forskolin (20 μM) (■———■) without and with GTPγS at the indicated concentrations. The assay was for 10 min at 25°C after a 15 min preincubation period with all reagents present except for labeled ATP.

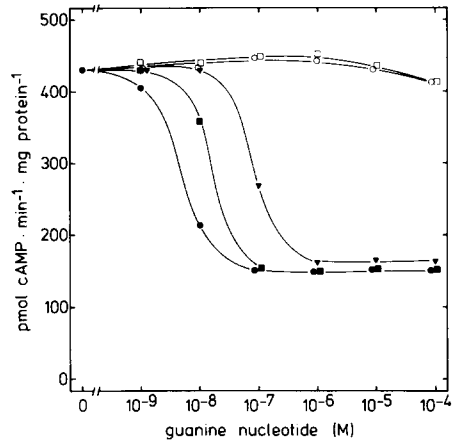

Fig. 2. Influence of various guanine nucleotides on the forskolin-stimulated human platelet adenylate cyclase. Adenylate cyclase activity was determined in human platelet membranes with 20 μM forskolin present without and with GTPγS (●———●), Gpp[NH]p (■———■), Gpp[CH_2]p (▼———▼), GTP (○———○) or GDPβS (□———□) at the indicated concentrations. The assay was for 10 min at 25°C after a 15 min preincubation period with all reagents present except for labeled ATP.

enzyme prostaglandin E_1 was increased by a factor of 2, while at higher concentrations of the stable GTP analog the activity was decreased. With any of the stimulants used, maximal inhibition by 60 to 70% was observed at 100 nM GTPγS. Since forskolin most efficiently stimulated the platelet adenylate cyclase and since this stimulated form was most sensitive to inhibition by GTPγS, in the following experiments shown, 20 μM forskolin was included in the reaction mixture.

Of the various guanine nucleotides studied, only the stable GTP analogs, GTPγS, Gpp[NH]p and Gpp[CH_2]p, inhibited the forskolin-stimulated platelet adenylate cyclase, with half-maximal inhibitions occurring at about 4, 20 and 100 nM, respectively (Fig. 2). With all of these analogs, the same maximal degree of inhibition was observed. In contrast to its stable analogs, GTP (up to 100 μM) did not inhibit the forskolin-stimulated platelet enzyme. At 0.1 to 10 μM GTP, even a small increase in activity was observed as reported before [11]. Similar to GTP, the stable GDP analog, GDPβS, which inhibits hormone-induced stimulations of the adenylate cyclase [26], did not inhibit the forskolin-stimulated platelet enzyme. When stimulation of the basal platelet adenylate cyclase activity by these guanine nucleotides was studied, the identical potency order was observed as for inhibition of the stimulated enzyme, i.e., GTPγS > Gpp[NH]p > Gpp[CH_2]p, as also observed in many other systems [3]. Furthermore, GTP and GDPβS did not stimulate basal activity (not shown).

Inhibition of the forskolin-stimulated platelet adenylate cyclase by GTPγS was a time-dependent process (Fig. 3). The time lag required for reaching final decreased activity was inversely related to the GTPγS concentration. When platelet membranes were pretreated with GTPγS for 15 min at 25°C, followed by an extensive washing of the membranes to remove free GTPγS, the adenylate cyclase appeared to be persistently inhibited (Table I). This persistent effect was maximal at 100 nM GTPγS, i.e., the concentration exhibiting maximal inhibition when added directly to the assay. A persistent inhibition was not observed when 1 mM EDTA was present in the pretreatment medium instead of 1 mM $MgCl_2$. As observed in other systems [12,15], inhibition of the forskolin-stimulated platelet adenylate cyclase by GTPγS was competitively inhibited by GDPβS

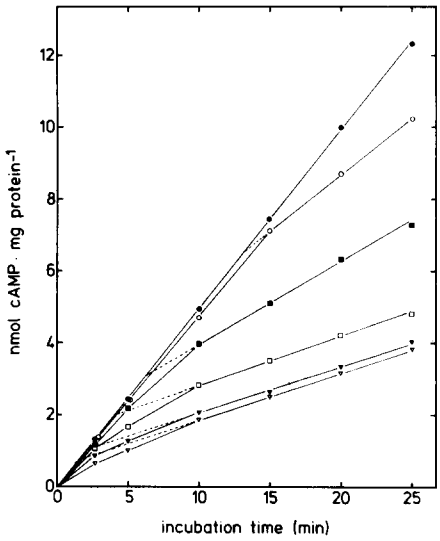

Fig. 3. Time course of the inhibitory effect of GTPγS on the forskolin-stimulated human platelet adenylate cyclase. Human platelet membranes were preincubated for 7 min at 25°C with 20 μM forskolin and the adenylate cyclase reaction mixture. Thereafter, the measurement of cyclic AMP accumulation was initiated by the addition of labeled ATP without (●———●) and with GTPγS at (nM): 3 (○———○); 10 (■———■); 30 (□———□); 100 (▼———▼); 1000 (▽———▽). The dashed lines are the extrapolations of the final rates of cyclic AMP accumulation.

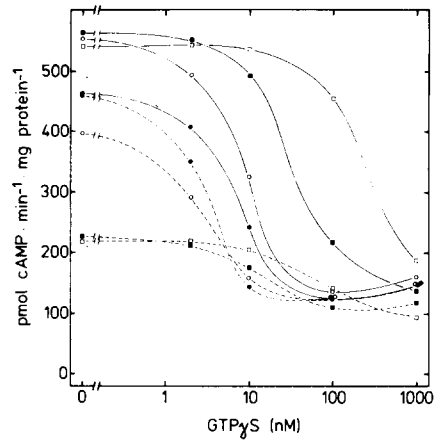

Fig. 4. Influence of GTP and epinephrine on the GTPγS-induced inhibition of the forskolin-stimulated platelet adenylate cyclase. In the presence of 20 μM forskolin, adenylate cyclase activity was determined in human platelet membranes without (●———●) and with GTPγS at the indicated concentrations in the absence and presence of GTP at various concentrations added without (solid lines) or with (dashed lines) 10 μM epinephrine. The concentrations of GTP are (μM); 0.1 (○); 1(■); 10 (□). The assay was for 10 min at 25°C after a 15 min preincubation period with all reagents present except for labeled ATP.

and was blocked in the presence of Mn^{2+} (at least 1 mM) (data not given).

The inhibitory hormone, epinephrine (10 μM), decreased the forskolin-stimulated platelet adenylate cyclase activity in a GTP-dependent manner (Fig. 4). Half-maximal and maximal inhibition by epinephrine was observed at 0.1 and 1 μM GTP, respectively. The maximal degree of adenylate cyclase inhibition induced by epinephrine plus GTP was identical to that observed with GTPγS

TABLE I

PERSISTENCE OF HUMAN PLATELET ADENYLATE CYCLASE INHIBITION BY GTPγS

Human platelet membranes (60 μg protein) were preincubated with either 1 mM $MgCl_2$ or 1 mM EDTA in the absence and presence of 0.1 μM GTPγS in 50 mM triethanolamine-HCl (pH 7.4) for 15 min at 25°C in a volume of 100 μl. Thereafter, 1 ml of ice-cold 10 mM triethanolamine-HCl (pH 7.4)/5 mM EDTA, was added and the membranes were pelleted by centrifugation for 10 min at 30000 × *g* and resuspended in the above buffer. This washing procedure was repeated twice. In the final pellets resuspended in 10 mM triethanolamine-HCl (pH 7.4), adenylate cyclase activity was determined with 20 μM forskolin in the absence and presence of 1 μM GTPγS for 10 min at 25°C after a 10 min preincubation period. Numbers in parentheses indicate adenylate cyclase inhibition (%) by GTPγS added to the incubation medium.

Addition to preincubation	Addition to incubation:	Adenylate cyclase activity (pmol cAMP/min per mg protein)	
		Forskolin	Forskolin + GTPγS
$MgCl_2$		487 ± 9	202 ± 7 (59)
$MgCl_2$ + GTPγS		227 ± 5	232 ± 6 (0)
EDTA		451 ± 8	197 ± 6 (56)
EDTA + GTPγS		447 ± 9	195 ± 5 (56)

alone. Epinephrine, which had no effect on the forskolin-stimulated activity in the absence of guanine nucleotides, caused a shift in the concentration-response curve of GTPγS to the left by 2- to 3-fold. At GTPγS concentrations causing maximal inhibition, i.e., at 0.1 and 1 μM, epinephrine had no effect. In the absence of epinephrine, GTP competitively inhibited adenylate cyclase inhibition by GTPγS. This effect of GTP to antagonize the GTPγS-induced inhibition was prevented when epinephrine was present in the incubation medium.

These measurements of adenylate cyclase activity were performed after a 15 min preincubation period with all reagents present except for labeled ATP. This procedure did not allow us to discriminate how epinephrine potentiated the inhibitory effect of GTPγS, by a diminishment of the lag period, by a decrease in final activity or by a combination of these two effects. Therefore, time course experiments were performed in which GTPγS was added to the enzyme together with the labeled ATP. The enzyme was preincubated for 7 min at 25°C with forskolin and the complete reaction mixture, since under the assay conditions

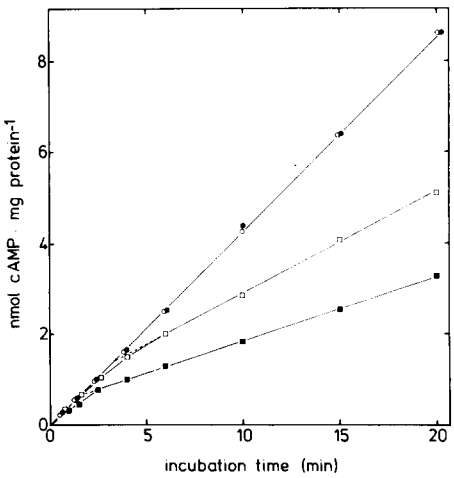

Fig. 5. Influence of epinephrine on the GTPγS-induced decrease in cyclic AMP accumulation. Human platelet membranes were preincubated for 7 min at 25°C with 20 μM forskolin and the adenylate cyclase reaction mixture without and with 10 μM epinephrine. Thereafter, the measurement of cyclic AMP accumulation was initiated by the addition of labeled ATP without and with 10 nM GTPγS. ○——○, No addition; ●——●, epinephrine; □——□, GTPγS; ■——■, epinephrine plus GTPγS.

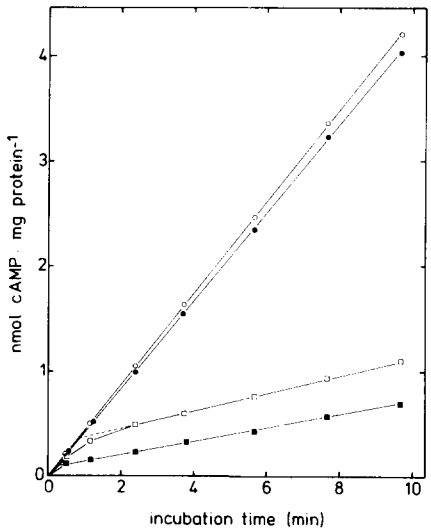

Fig. 6. Influence of epinephrine on the decrease in cyclic AMP accumulation induced by GTPγS at a maximally effective concentration. The conditions and symbols are identical to those described in Fig. 5. Epinephrine was 10 μM and GTPγS was 1 μM.

used the forskolin-stimulated cyclic AMP formation was linear only after 5 min of incubation (not shown). Epinephrine, when studied, was also included in the preincubation medium, since it was without effect on the enzyme activity when guanine nucleotides were not present. As shown in Figs. 5 and 6, epinephrine had two effects on the GTPγS-induced inhibition of cyclic AMP formation. At 10 nM GTPγS, 10 μM epinephrine caused a further decrease in final activity by 40% and diminished the lag period required for reaching final activity by a factor of 2 (Fig. 5). At 1 μM GTPγS, a maximally effective GTPγS concentration, epinephrine had no effect on the final decreased activity but caused a marked diminishment of the lag period by a factor of 3 (Fig. 6). Thus, the epinephrine-induced increase in the inhibitory potency of GTPγS observed after 15 min of preincubation (see Fig. 4) was due to a decrease in final activity.

A concentration-response curve for this effect of epinephrine to amplify the GTPγS-induced inhibition of the forskolin-stimulated platelet adenylate cyclase is shown in Fig. 7. In the absence of GTPγS, epinephrine (up to 10 μM) had almost no effect. With increasing concentrations

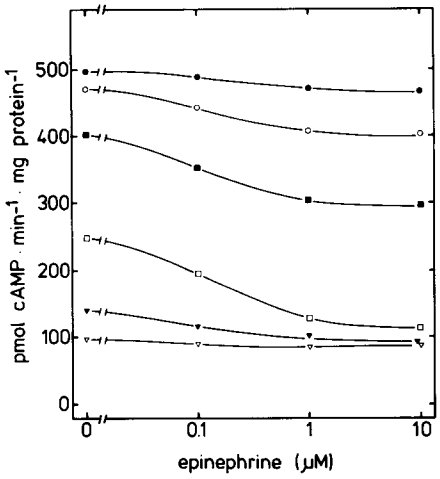

Fig. 7. Amplification of the GTPγS-induced inhibition of the platelet adenylate cyclase by epinephrine. In the presence of 20 μM forskolin, adenylate cyclase activity was determined in human platelet membranes without and with epinephrine at the indicated concentrations in the absence (●———●) and presence of GTPγS at (nM): 1 (○———○); 3 (■———■); 10 (□———□); 30 (▼———▼); 100 (▽———▽). The assay was for 10 min at 25°C after a 15 min preincubation period with all reagents present except for labeled ATP.

of GTPγS, the additional inhibitory action of epinephrine became more and more evident. At 30 nM GTPγS, a suboptimal concentration, the effect of epinephrine was very small, and at 100 nM GTPγS, a maximally inhibitory concentration, epinephrine did not cause a further decrease in activity, as also shown before (see Figs. 4 and 6).

Discussion

The data presented in this communication show that stable GTP analogs, which stimulate basal adenylate cyclase activity, can inhibit stimulated forms of the human platelet enzyme. Similar data have been described before in several different tissues including human platelets [5–15]. In contrast to the data reported for rat striatal membranes [12], inhibition of the platelet adenylate cyclase by stable GTP analogs was a time-dependent process. Furthermore, the presence of forskolin was apparently not required in the preincubation medium in order to see the persistent inhibition of the platelet enzyme, whereas it was not seen when EDTA was present. In cyc$^-$ variants of S49 lymphoma cells, which are deficient in the stimulatory coupling component, N_s [27], we observed an identical pattern of adenylate cyclase inhibition by stable GTP analogs as shown herein for human platelet adenylate cyclase [15]. In those cells, the enzyme was stimulated by forskolin or by purified, preactivated N_s-protein.

With regard to the mechanism of adenylate cyclase inhibition by stable GTP analogs, it has been suggested that these nucleotides interact with the inhibitory guanine nucleotide site, N_i, mediating hormone-induced adenylate cyclase inhibition [12–15]. The similarity between adenylate cyclase stimulation and inhibition by stable GTP analogs, furthermore, suggested that the two coupling components, N_s and N_i, are activated by similar mechanisms. N_s is apparently activated either by the combined action of a hormone and GTP or by stable GTP analogs [3]. The activation of N_s by stable GTP analogs is time-dependent and resistant to washing of the membranes and can be accelerated and amplified by stimulatory hormones. Accordingly, similar reactions should be observed for the inhibitory site, N_i. Therefore, it was studied whether the inhibitory hormone, epinephrine, can accelerate and/or amplify the inhibitory actions of stable GTP analogs on the adenylate cyclase in human platelet membranes. It is shown herein that epinephrine accelerates the inhibitory action of the stable GTP analog, GTPγS. This was observed with submaximally and maximally effective concentrations of GTPγS. Furthermore, epinephrine amplified the adenylate cyclase inhibition by GTPγS. This amplification was observed only at submaximally inhibitory concentrations of GTPγS and was most pronounced at GTPγS concentrations causing half-maximal inhibition. At maximally effective GTPγS concentrations, epinephrine caused only acceleration but not an amplification of the inhibitory action of GTPγS. This ineffectiveness of epinephrine and the apparent high affinity of N_i for the stable GTP analogs readily explain why in previous studies [28] not an amplification but an apparent blockade of the epinephrine-induced inhibition by stable GTP analogs was observed. In those studies, the stable GTP analog, Gpp[NH]p, was used in concentrations from 1 to 100 μM which are 10- to 1000-fold higher than the concentration

required for full activation of N_i by this analog.

The regulation of human platelet adenylate cyclase by stimulatory and inhibitory hormones and by guanine nucleotides therefore appears to be best explained by the following mechanisms. Whereas prostaglandin E_1 and other stimulatory prostaglandins increase the adenylate cyclase activity through a N_s-mediated process, epinephrine via α_2-adrenoceptors causes adenylate cyclase inhibition through an N_i-mediated mechanism. For both hormonal actions, the presence of GTP is required [23,29]. Both GTP-dependent hormonal actions can be inhibited by the stable GDP analog, GDPβS [30]. N_s and N_i, can also be activated by stable GTP analogs such as GTPγS and Gpp[NH]p. Due to their resistance to hydrolysis by GTPases, both actions of stable GTP analogs, i.e., activation of N_s and N_i are resistant to washing of the membranes, and therefore, persistent activation or inhibition of the adenylate cyclase is observed. The activation process of both N_s and N_i by stable GTP analogs is time-dependent and can be accelerated and amplified by either prostaglandin E_1 or epinephrine.

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