Stimulation of Catecholamine-sensitive Adenylate Cyclase by 5'-Guanylyl-Imidodiphosphate

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SUMMARY

The GTP analog 5'-guanylyl-imidodiphosphate (Gpp(NH)p) caused marked stimulation of basal and catecholamine (isoproterenol)-sensitive adenylate cyclase from canine myocardium, frog erythrocytes and rat paraovarian fat. The combination of Gpp(NH)p (10^{-4} M) and isoproterenol (10^{-4} M) produced activation of adenylate cyclase equal to (fat) or significantly greater than (heart, erythrocytes) that stimulated by fluoride ion. The great activity of Gpp(NH)p was due not to its greater resistance to hydrolysis than GTP or to inhibition of ATP hydrolysis, since an ATP regenerating system was employed in the adenylate cyclase assays, and no significant hydrolysis of ATP or GTP occurred.

GTP caused stimulation of adenylate cyclase in myocardial membranes, was virtually without effect in frog erythrocytes and resulted in inhibition in adipose membranes. Despite the much greater enzyme activation by Gpp(NH)p than GTP, the affinity of GTP for the nucleotide regulatory sites on the enzyme was greater. GTP potently and competitively antagonized the greater enzyme stimulation by Gpp(NH)p. Adenylate cyclase stimulation by any concentration of Gpp(NH)p was 50% inhibited by a 10-fold lower concentration of GTP. The concentration of Gpp(NH)p necessary for half-maximal stimulation was doubled by 10^{-4} M GTP. Ability to competitively antagonize stimulation by Gpp(NH)p provided a convenient way of assessing the relative affinities of nucleotides for the regulatory sites. These affinities were GTP > GTP > GMP > ITP with UTP and CTP either very weak or inert.

Since GTP and other nucleotides are capable of causing only partial activation of the enzyme, comparison of their affinities for the nucleotide regulatory sites on the basis of ability to stimulate adenylate cyclase may be misleading. This is particularly true in frog erythrocyte membranes where the guanine nucleotides have almost no intrinsic activity although they may have high affinity for the sites.

The marked stimulation of adenylate cyclase by Gpp(NH)p appears to be due to an increase in the V_max of the enzyme rather than to a change in the affinity for substrate, MgATP, or divalent cation (Mg^{2+}).

Investigations from several laboratories have documented that guanine nucleotides, especially GTP, exert a regulatory influence on adenylate cyclase in a wide variety of tissues (1–9). With the exception of reports of an inhibitory effect in fat cell membranes (2), the interaction of the nucleotides with regulatory sites on adenylate cyclase has generally resulted in stimulation of basal and hormone-sensitive enzyme activity and an inhibition of fluoride-stimulated activity. Stimulation of hormone-sensitive adenylate cyclase has often been greater than that of basal enzyme activity.

Comparisons of the ability of nucleotides to interact with these regulatory sites have generally been based on their relative activity in stimulating the adenylate cyclase. In the course of recent investigations of the binding of [H]GTP to solubilized myocardial adenylate cyclase preparations we observed that the GTP analog Gpp(NH)p caused much greater activation of the enzyme than GTP, though its affinity for the regulatory sites as assessed by binding studies was lower than that of GTP. This suggested that GTP might be capable of occupying the regulatory sites on the enzyme without necessarily causing activation. This situation would be analogous to that of a partial agonist drug which, although it may have very high affinity for a receptor, may be capable of causing only partial activation. The studies to be reported here suggest just such a situation with regard to the nucleotide regulatory sites on adenylate cyclase from three different tissues (canine myocardium, rat paraovarian fat, and frog erythrocyte).

Gpp(NH)p, GTP, and other nucleotides appear to interact with the same sites on the enzyme. Gpp(NH)p causes maximal activation of basal and catecholamine-sensitive activity through these sites (activity greater than that stimulated by fluoride ion), whereas GTP is capable of producing only partial activation. However, the affinity of GTP for the regulatory sites is higher than that of Gpp(NH)p as shown by its ability to potently

1 The abbreviations used are: Gpp(NH)p, 5'-guanylyl-imidodiphosphate; Gpp(CH)p, 5'-guanylyl-α,γ-methylene-diphosphonate; cyclic AMP, cyclic adenosine 3':5'-monophosphate.

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and apparently competitively antagonize the greater enzyme stimulation by Gpp(NH)p. Gpp(NH)p activation of the enzyme appears to be caused by an increase in the \( V_{\text{max}} \), rather than an effect on \( K_m \) for substrate MgATP or MgATP for (MgGTP).

**MATERIALS**

1-Isoproterenol bitartrate, d,l-propanolol, GTP, GDP, GMP, ITP, UTP, CTP, myokinase, phosphoethanolpyruvate, pyruvate kinase, cyclic AMP, and ATP were purchased from Sigma. Gpp(NH)p was from I. C. N., Gpp(CH$_2$)p was from Miles. [a-32P]ATP (1 to 10 Ci per mmole) was from New England Nuclear Co. Cyclic [3H]AMP was from Schwarz-Mann. Dowex 50W-X2 (200 to 400) mesh was from Bio-Rad and alumina, neutral grade, was from Nutritional Biochemicals.

**METHODS**

**Tissue Samples**

Left Ventricular Myocardiun—Myocardium was obtained from adult mongrel dogs (20 to 40 kg) killed by injection of intravenous pentobarbital. The tissue (0.5 g), minced with scissors, was suspended in 10 ml of cold 0.25 M sucrose-0.005 M Tris- HCl, pH 7.4. The tissue mince was disrupted by a 10-s exposure to a "Tissuemizer" (Teknor Co.) operating at half-maximal speed. The thick slurry obtained was further homogenized with 10 strokes of a motor-driven Teflon-tipped pestle. The tissue homogenate was passed through a single layer of cheesecloth and then centrifuged at 12,000 \( \times g \) for 10 min in a Sorvall RC2B centrifuge at 2\(^\circ\)C. The pellet was resuspended in the original volume of sucrose buffer and recentrifuged at 12,000 \( \times g \) for 10 min. The washing procedure was repeated three or four times. Ultimately the pellet was resuspended and homogenized in a volume of 75 mM Tris- HCl, pH 7.4, and 25 mM MgCl$_2$, equal to the original homogenerate volume. Membrane fractions were immediately assayed for enzyme activity.

Isolated Fat Cells—Cells were prepared from the parovarian fat from 200- to 325-g Charles River (CD strain) rats fed ad libitum. Animals were killed by cervical dislocation, the paraovarian fat from 200- to 225-g Charles River (CD strain) rats fed ad libitum. The fat was minced, and membranes were prepared essentially as described by Czech and Lynn (10). In brief, the minced tissue was divided into 4- to 5-g portions and placed in plastic bottles containing 8 to 10 ml of 5% bovine serum albumin in "buffer" (128 mM NaCl, 1.8 mM CaCl$_2$, 4.5 mM MgSO$_4$, 5.2 mM KCl, and 10 mM NaHPO$_4$, pH 7.4) with 1 mg of crude collagenase type I (Clos- tridium histolyticum) Worthington per g tissue and was incubated 60 min at 37\(^\circ\)C. After digestion, cells were filtered through cheesecloth and washed twice with albumin buffer, collected by centrifugation and added to a prechilled glass homogenizer with 25 ml of ice-cold "buffer," and homogenized (Teflon pestle) with seven up-and-down strokes. The homogenate was centrifuged at 13,000 \( \times g \) for 15 min. The resulting pellet was washed by resuspension and centrifugation in ice-cold 0.25 M sucrose, 0.005 M Tris- HCl, pH 7.4, three times and finally suspended in 75 mM Tris- HCl, pH 7.4, and 25 mM MgCl$_2$, by homogenization.

Frog Erythrocytes—Blood from cold- or warm-adapted grass frogs (Carolina Biological, Burlington, N. C.) was collected and the red cells were washed three times with 0.15 M saline. Cells were lysed in 5 mM Tris- HCl, pH 8.1, buffer and the membranes centrifuged at 18,000 \( \times g \) for 15 min. The lysis process was repeated three times. Membranes were finally suspended in 75 mM Tris- HCl and 25 mM MgCl$_2$, pH 8.1, by homogenization.

**Adenyate Cyclase Assay**

Assay was performed using a modification of the method of Krishna et al. (11). Assays were performed in a volume of 50 \( \mu l \) which contained Tris- HCl buffer, 30 mM (pH 7.4); MgCl$_2$, 10 mM; cyclic AMP, 0.1 mM; ATP, 1.5 mM; GTP, 0.1 mM; [gamma-32P]ATP, 1 to 2 \( \times 10^{6} \) cpm per ml; phosphoethanolpyruvate, 5 mM; pyruvate kinase, 40 \( \mu g \) per ml; and myokinase, 20 \( \mu g \) per ml. Incubations were for 10 min at 37\(^\circ\)C and were stopped by addition of 1 ml of a solution containing cyclic [PH]AMP (15,000 cpm per ml), ATP, 100 \( \mu g \); and cyclic AMP, 50 \( \mu g \). Cyclic [gamma-32P]AMP that was formed was isolated by chromatography either on Dowex AG 30W-X2 as previously described (12) or on neutral alumina as described by Ramachandran (13). Recovery was 50% on Dowex and 70 to 75% on the alumina columns. Samples were added to 15 ml of scintillation fluid (14), and counted in a Packard liquid scintillation spectrometer. All results were correct for protein recovery on the basis of recovery of cyclic [3H]AMP.

For all membrane preparations it was determined that reactions were linear over the course of the 10-min incubation. Enzyme activity was directly proportional to protein concentration over the range of proteins (10 to 70 \( \mu g \)) used in the assays. Membranes were prepared fresh each day for each experiment.

**Chromatography of Nucleotides**

To assess the extent of degradation of nucleotides by membranes during adenylate cyclase incubations, the following procedures were employed. Aliquots of each of the membranes were incubated with [gamma-32P]ATP and [3H]GTP in the presence of the regenerating system and other reagents as described above for adenylate cyclase assays. At the completion of a 10-min incubation, nucleotides were applied to polyethyleneimine (PEI) cellulose- plate (Brinkman) which were developed with 1 M LiCl-1 M formic acid as described by Randerath and Randerath (15). In all cases, >97% of either ATP or GTP appeared to be undegraded nucleotide at the end of such incubations. Similar procedures were employed to assess conversion of [3H]GMP or [3H]GDP to [3H]GTP under standard incubation conditions. During the 10-min incubations, 32% of GMP and 85% of added GDP were converted to GTP.

Protein was determined by the method of Lowry et al. (16).

**RESULTS**

Catecholamine-sensitive adenylate cyclase was found in the myocardial, adipose, and erythrocyte membranes (17). Gpp(NH)p, previously reported to stimulate adenylate cyclase in fat membranes (2), stimulated basal and catecholamine-sensitive adenylate cyclase in each of the three types of membranes tested in these studies. In each case, Gpp(NH)p caused greater stimulation of basal enzyme activity than Gpp(CH$_2$)p or GTP (Fig. 1, A to C). Gpp(CH$_2$)p was relatively weak in erythrocytes and fat and much more active in heart membranes. The effects of GTP varied. GTP caused almost no stimulation in the erythrocyte membranes, but did stimulate the enzyme in heart tissue. In adipose membranes, GTP produced up to a 65% inhibition of basal and catecholamine-stimulated activity (as previously reported by Harwood et al. (2)). The effects of Gpp(NH)p on catecholamine-sensitive activity were even more striking than those on basal activity (Fig. 1, A to C). In each tissue Gpp(NH)p (10$^{-4}$ M) plus isoproterenol (10$^{-4}$ M) caused marked stimulation of the enzyme. This combination was synergistic in erythrocyte and cardiac membranes and appeared to be additive in adipose membranes (Fig. 2, A to C). Gpp(NH)p plus isoproterenol caused greater stimulation of the enzyme than 10 mM NaF (a maximal stimulatory concentration) in heart and erythrocyte membranes. Stimulation by isoproterenol plus Gpp(NH)p was 8-fold (over basal activity) in heart, 10-fold in adipose membranes, and 100-fold in erythrocyte membranes. By comparison, stimulation by fluoride was 5.5-fold in heart, 10-fold in adipose membranes, and 25-fold in erythrocyte membranes.

Of further note were the effects of propranolol, which completely blocked the augmented effects of isoproterenol seen in the presence of Gpp(NH)p. This finding suggests that the augmented stimulation of adenylate cyclase by isoproterenol observed in the presence of Gpp(NH)p is mediated by a typical \( \beta \)-adrenergic receptor.

In heart and adipose membranes (10$^{-4}$ M Gpp(NH)p caused a 20 to 30% reduction in activity stimulated by 10 mM F$^{-}$, whereas, in the erythrocyte membranes, it caused essentially no reduction in fluoride stimulated activity (data not shown).

The consistently greater activity of Gpp(NH)p than GTP on
the adenylate cyclase was not due to the resistance of the analog to hydrolysis, nor to protection of substrate ATP from hydrolysis. In the presence of the regenerating system used in these studies, no significant destruction of ATP or GTP could be detected during a 10-min incubation. (This was determined by chromatographing the labeled nucleotides before and after incubation with aliquots of each of the membranes, as described under “Methods”).

As noted in Fig. 1, enzyme activation by GTP in heart and erythrocyte membranes did not approach that achieved by...
Fig. 3. Competitive inhibition of Gpp(NH)p stimulation of adenylate cyclase by GTP in A, myocardial membranes; B, erythrocyte membranes; and C, adipose membranes. In each panel the uppermost curve (■—■) is enzyme activity in the presence of 10^{-3} to 10^{-1} M Gpp(NH)p, whereas the lowest curve (○—○) is activity in the presence of 10^{-7} to 10^{-3} M GTP. Each of the other curves represents enzyme activity in the presence of 10^{-7} to 10^{-3} or 10^{-4} M Gpp(NH)p plus a fixed concentration of GTP which is indicated to the right of the curve. Each value is the mean of six determinations from three separate experiments.

Gpp(NH)p even at very high concentrations of GTP. Two possible explanations could account for this observation. Gpp(NH)p might interact with additional sites on the enzyme with which GTP does not interact. Alternatively, GTP and Gpp(NH)p might interact with the same sites. In this case, the analog would have a greater intrinsic activity (18) or ability to stimulate the enzyme. This might be true even though GTP had a higher affinity for the sites. The latter explanation is consistent with the data in Fig. 3, A to C. In each of the membranes, GTP potently and competitively antagonized the greater stimulation of the enzyme by Gpp(NH)p. Stimulation by any concentration of Gpp(NH)p was about 50% inhibited by a 10-fold lower concentration of GTP (slightly less inhibition in fat). GTP, 10^{-7} M, generally doubled the concentration of Gpp(NH)p necessary for half-maximal response. Double-reciprocal plots of the data in Fig. 3 also show competitive inhibition. Thus, GTP is capable of causing only partial activation of the enzyme through interaction with the regulatory sites. Nonetheless, its affinity for these sites is greater than that of Gpp(NH)p which appears to be a maximal activator.

The ability of several nucleotides to stimulate the adenylate cyclase and to antagonize the greater stimulation by Gpp(NH)p in each of the membranes is shown in Fig. 4, A to C. An estimate of the affinity of each nucleotide for the regulatory sites could be made by comparing their abilities to inhibit the activation of the enzyme by Gpp(NH)p. Guanine nucleotides were most potent in the order GTP > GDP > GMP. ITP interacted weakly and the pyrimidine nucleotides UTP and CTP were almost ineffective. Since GMP was converted partially and GDP largely to GTP during the incubations (in the presence of the regenerating system) much of the observed activity of these nucleotides may have been due to conversion to GTP.

As noted above, Gpp(NH)p, especially in the presence of isoproterenol, caused maximal stimulation of adenylate cyclase. In order to further study the mechanism by which this marked augmentation of enzyme activity was effected a kinetic analysis of the effect of Gpp(NH)p on the enzyme was undertaken (19). The results of these experiments are presented in Fig. 5, A to C. Enzyme activity (heart) in the presence or absence of 10^{-4} M Gpp(NH)p was measured at several different ATP concentrations (0.1 to 6.0 mM) and a number of different Mg^{2+} concentrations (1.25 to 40 mM). Gpp(NH)p stimulated the enzyme at all ATP concentrations and at all Mg^{2+} concentrations, even those above saturation. This stimulation was not accompanied by a significant alteration in $K_M$ for MgATP or $K_a$ for Mg^{2+}. (Comparable results were obtained in erythrocyte membranes.) Thus, the marked stimulatory effects of Gpp(NH)p are likely due to an increase in $V_{max}$ of the enzyme.

**DISCUSSION**

Stimulation of adenylate cyclase by Gpp(NH)p in concert with the $\beta$-adrenergic catecholamine isoproterenol produces maximal activation of the enzyme, activity equal to (fat) or greater than (heart, erythrocyte) that elicited by fluoride ion. The data strongly suggest that the effects of this GTP analog are produced via interaction with the same nucleotide regulatory sites with which other nucleotides have been shown to interact. Activity of Gpp(NH)p was greater than that of GTP in all cases. GTP, however, potently and competitively inhibited the greater enzyme activation by Gpp(NH)p. The competitive nature of this inhibition is indicated by the parallel slopes of the dose response curves in Fig. 3 and also by double-reciprocal plots (not shown). The significance of this observation is that although GTP possesses high affinity for the nucleotide regulatory sites on adenylate cyclase, it is capable of only partially activating the enzyme (heart, erythrocyte) or inhibiting it (fat). Conversely, Gpp(NH)p, which has significantly lower affinity produces full activation. It has not been generally appreciated that nucleotides can interact with these regulatory sites without necessarily producing a direct measurable effect on the enzyme (1-9). Thus, in formulating “order of potencies” of nucleotides for these sites, ability of the nucleotides to stimulate the enzyme has generally been used exclusively as the measure of affinity for the sites. Much of this previously reported data will now have to be re-evaluated in the light of the current findings. For example, in our experiments, guanine nucleotides such as GTP, GDP, and GMP caused little or no stimulation of adenylate cyclase in frog erythrocytes. The same was true of the pyrimidine nucleotides UTP and CTP.

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hypothesis that affinity of GTP for the nucleotide regulatory sites is always very high but that its intrinsic activity or ability to cause enzyme activation may vary greatly from tissue to tissue (sometimes being close to 0 as in frog erythrocyte membranes) may help to unify a number of disparate observations concerning effects of guanine nucleotides on adenylate cyclase.

As with studies of hormone-receptor interaction, the most direct way to assess affinity of nucleotides for these regulatory sites would be to perform direct binding studies with radioactively labeled nucleotides. The unavailability of purified preparations of adenylate cyclase with which to perform such binding studies, however, has retarded progress in this area. Nonetheless, with partially purified preparations of solubilized myocardial adenylate cyclase we have recently performed binding studies with \(^{3}H\)GTP.\(^3\) In these preparations, Gpp(NH)p competed for the \(^{3}H\)GTP binding sites more weakly than GTP, although it caused much more enzyme stimulation. As has been noted, however, GTP potently inhibits activation by Gpp(NH)p with an apparently higher affinity for the sites than Gpp(NH)p. Thus, both the binding and the enzyme studies indicate the higher affinity of GTP for the regulatory sites.

In addition to stimulating adenylate cyclase, Harwood et al. (2) and Cryer et al. (20) have reported that under certain circumstances GTP inhibits basal and hormone-sensitive fat cell adenylate cyclase. We, too, observed inhibition of fat cell adenylate cyclase by GTP, GDP, and ITP. Each of these nucleotides, at 10^{-4} M, also antagonized enzyme stimulation by Gpp(NH)p (Figs. 3C and 4C). All of our experiments were performed in the presence of a regenerating system and, as pointed out by Harwood et al. and confirmed in these studies, the observed effects of GDP may well have been due to conversion to GTP (2). These authors also stated that “because of the inhibitory effects of GTP on basal activity . . . it is dif-

\(^{3}\) R. J. Lefkowitz, manuscript in preparation.
Fig. 5. Kinetic effects of Gpp(NH)p on myocardial adenylate cyclase A, at varying Mg2+ concentrations; B and C, at varying ATP concentrations. In A, basal and Gpp(NH)p-stimulated enzyme activity were determined at several fixed ATP concentrations (0.1, 0.5, 1.5, and 6.0 mM) which are indicated to the right of the curves, over a range of Mg2+ concentrations from 1.25 to 40 mM. Mg2+ was present as MgCl2. In B and C, the data are replotted (19) as a function of ATP concentration at fixed Mg2+ concentrations (5 to 40 mM Mg2+ in B, 1.25 to 2.5 mM Mg2+ in C). Gpp(NH)p concentration, when present, was 10^{-4} M. All values are means of four determinations from two separate experiments.

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REFERENCES
15. RANDETH, K., AND RANDERATH, E. (1964) J. Chromatogr. 16, 111-125
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