Specificity for Guanine Nucleotide Activation and Stabilization of Rabbit Cardiac Adenylate Cyclase

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These studies examined the structural specificity for guanine nucleotide-facilitated hormonal activation and guanine nucleotide stabilization of cardiac adenylate cyclase. 1. The phosphonate analogues of GTP, p[CH₂]ppG (guanosine 5'-[βγ-methylene]triphosphate) and pp[CH₂]pG (guanosine 5'-[αβ-methylene]triphosphate), were the most effective activators of adenylate cyclase. Other nucleotides producing significant activation ($P < 0.01$) were, in decreasing order of activation: ITP, GDP, GMP, GTP, XTP, CTP, p[NH]ppG (guanosine 5'-[βγ-imido]triphosphate), dGTP and 2'-O-methyl-GTP. Guanosine, cyclic GMP, UTP and ppppG (guanosine tetraphosphate) had no effect, and 7-methyl-GTP caused a decrease in the activity. 2. Preincubation of membranes at 37°C for 15 min before assay at 24°C produced an 80% decrease in adenylate cyclase activity, and preincubation with p[CH₂]ppG and pp[CH₂]pG protected and resulted in a net increase in activity. Other nucleotides that completely or partially preserved activity in decreasing order of effectiveness were p[NH]ppG, GDP, GTP, dGTP, ITP, ppppG, 2'-O-methyl-GTP, GMP, CTP and XTP. Several compounds had no effect, including guanosine, cyclic GMP and UTP, whereas preincubation with 7-methyl-GTP produced a further decrease ($P < 0.05$) in activity. 3. The concentration-dependence for activation and stabilization by the naturally occurring guanine nucleotides was examined in the absence of a regenerating system and revealed GMP to have no stabilizing effect and to be less potent than either GDP or GTP in activating adenylate cyclase. 4. A significant correlation ($r = 0.90$) was found between the properties of activation and stabilization for the compounds examined. These findings are consistent with there being a single nucleotide site through which both the activation and stabilization of adenylate cyclase are mediated.

Guanine nucleotides have come to be accepted as important modifiers of the adenylate cyclase system. They alone activate adenylate cyclase, and hormone increases the rate of nucleotide activation (Salomon *et al.*, 1975; Cuatrecasas *et al.*, 1975; Pfeuffer & Helmreich, 1975; Jacobs *et al.*, 1976; Rendell *et al.*, 1977). The affinity of hormone binding to membrane preparations is decreased (Rodbell *et al.*, 1971; Glossman *et al.*, 1974) or increased (Spiegel & Aurbach, 1974) by guanine nucleotide, and in the hepatic system this may represent a functionally distinct nucleotide site from that involved in activation (Lad *et al.*, 1977). In addition to these effects, guanine nucleotides increase the stability of adenylate cyclase (Rodbell *et al.*, 1971;

Harwood *et al.*, 1973; Cuatrecasas *et al.*, 1975; Snyder & Drummond, 1978; Glynn *et al.*, 1978). This activity has been found to be relatively labile in membrane preparations from a variety of tissues (Pilkis & Johnson, 1974; Cuatrecasas *et al.*, 1975; Neer, 1976; Snyder & Drummond, 1978; Glynn *et al.*, 1978; Ross *et al.*, 1978). The characterization of the effects that are mediated through single or multiple guanine nucleotide sites is incomplete. Evidence that guanine nucleotide binding to membranes greatly exceeds the potential number of sites associated with adenylate cyclase (Pfeuffer & Helmreich, 1975; Pfeuffer, 1977) indicates the difficulty of identifying the number of functionally relevant sites.

In the present study the guanine nucleotide structural requirements for both activation in the presence of hormone and stabilization of cardiac adenylate cyclase were examined to determine whether the structural requirements for these two effects were similar or distinct. Cardiac adenylate cyclase can be

Abbreviations used: p[CH₂]ppG, guanosine 5'-[βγ-methylene]triphosphate; pp[CH₂]pG, guanosine 5'-[αβ-methylene]triphosphate; p[NH]ppG, guanosine 5'-[βγ-imido]triphosphate; ppppG, guanosine tetraphosphate; ppGpp, guanosine 5'-diphosphate 3'-diphosphate; pppGpp, guanosine 5'-triphosphate 3'-diphosphate.

optimally activated by guanine nucleotide, and the stability of this activity at 37°C is greatly increased by guanine nucleotide (Snyder & Drummond, 1978). The activation and stabilization of adenylate cyclase by guanine nucleotide are two processes that can be examined separately by appropriate experimental protocol. A number of studies have examined the structural requirements for guanine nucleotide activation (Harwood *et al.*, 1973; Salomon *et al.*, 1975; Pfeuffer & Helmreich, 1975; Pfeuffer & Eckstein, 1976; Spiegel *et al.*, 1977; Londres *et al.*, 1977; Kimura & Nagata, 1977), but little information is available on the structural requirements for nucleotide-mediated stabilization. Nucleotides examined in this study include compounds with modifications in the base and ribose moieties, variation in the degree and position of phosphorylation and altered phosphate ester bonds.

Materials and Methods

Cyclic [G-³H]AMP (ammonium salt, 37 Ci/mmol) and [2,8-³H]ATP (tetrasodium salt, 25 Ci/mmol)

were obtained from New England Nuclear. Guanosine, cyclic GMP, GMP, GDP, GTP, dGTP, 2'-O-methyl-GTP, ppppG, ppGpp (MSI), pppGpp (MSII), pp[CH₂]pG, p[CH₂]ppG, p[NH]ppG, 7-methyl-GTP, XTP, ITP, UTP and CTP were obtained from P.L. Biochemicals. XTP, ITP, UTP and CTP were found by high-pressure liquid chromatography to have no detectable contamination by GTP (less than 0.4%). The source of other materials was as previously reported (Snyder & Drummond, 1978).

Heart membranes were prepared as previously described (Snyder & Drummond, 1978) from 8–12-week-old New Zealand male rabbits.

Adenylate cyclase was assayed essentially as previously described (Snyder & Drummond, 1978) during a 10 min incubation at 24°C with 275–325 µg of membrane protein in a final volume of 0.150 ml containing 0.05 M-sucrose, 1 mM-3-isobutyl-1-methyl-xanthine, 10 or 25 mM-MgCl₂, 0.5 mM-[2,8-³H]ATP (approx. 20 mCi/mol), 20 mM-phosphoenolpyruvate, 25 µg of pyruvate kinase, 5.6 mM-KCl and 40 mM-Tris/HCl, pH 7.5. Cyclic AMP was isolated by the method of Salomon *et al.* (1974).

Table 1. *Specificity of guanine nucleotide-facilitated hormonal activation of cardiac adenylate cyclase*
Heart membranes were preincubated for 15 min at 24°C in the absence and presence of isoproterenol with various nucleotides (1 mM). Adenylate cyclase was then assayed at 24°C under standard conditions with 10 mM-MgCl₂. In the absence of any additions, control activity was 77.5 ± 19.1 (*n* = 10) pmol/min per mg of protein and in the presence of isoproterenol but no further additions 95.0 ± 15.9 (*n* = 9) pmol/min per mg of protein. Results are expressed as means ± s.d. for the numbers of determinations given in parentheses and are activities relative to that in the absence of any additions. Significance of difference from respective controls: ***P* < 0.01; ****P* < 0.001.

Additions	Relative activity	
	No isoproterenol	0.1 mM-Isoproterenol
None	1.00 ± 0.24 (10)	1.41 ± 0.23 (9)
(a) Modified phosphate		
Guanosine	1.30 ± 0.12 (3)	1.62 ± 0.23 (4)
Cyclic GMP	1.00 ± 0.31 (3)	1.45 ± 0.27 (4)
GMP	1.59 ± 0.49 (3)	2.51 ± 0.63 (4)***
GDP	1.27 ± 0.18 (3)	2.58 ± 0.73 (4)***
GTP	1.20 ± 0.18 (3)	2.41 ± 0.50 (4)***
ppppG	0.69 ± 0.10 (3)	1.25 ± 0.42 (4)
ppGpp (MSI)	1.08 (1)	0.89 (1)
pppGpp (MSII)	0.95 (1)	0.83 (1)
p[NH]ppG	1.17 ± 0.15 (3)	2.06 ± 0.18 (3)***
p[CH ₂]ppG	1.68 ± 0.70 (3)**	5.76 ± 2.64 (4)***
pp[CH ₂]pG	1.78 ± 0.51 (3)***	5.05 ± 1.91 (5)***
(b) Modified ribose		
dGTP	1.14 ± 0.18 (3)	2.00 ± 0.37 (4)***
2'-O-Methyl-GTP	0.91 ± 0.14 (3)	1.99 ± 0.28 (4)**
(c) Modified base		
7-Methyl-GTP	0.88 ± 0.21 (3)	1.03 ± 0.17 (4)
XTP	0.98 ± 0.29 (3)	2.25 ± 0.79 (4)***
ITP	1.16 ± 0.33 (3)	2.63 ± 0.73 (4)***
UTP	0.94 ± 0.12 (3)	1.41 ± 0.13 (4)
CTP	1.29 ± 0.22 (3)	2.22 ± 0.29 (4)***

Results

Specificity for guanine nucleotide-facilitated hormone activation

Previous studies have shown that adenylate cyclase from rabbit heart membranes is nearly maximally activated during a 10min incubation at 24°C in the presence of p[NH]ppG and isoproterenol (Snyder & Drummond, 1978). The activated state had an increased $V_{\max}$, and a decreased saturation requirement for Mg^{2+} from approx. 50 to 10mM. In the present study, assays were performed in the presence of 10mM- Mg^{2+} to optimize the potential difference between the basal and activated states. Rabbit heart membranes were incubated at 24°C for 15min in the absence or presence of DL-isoproterenol (0.1mM) with various nucleotides (1.0mM), and then assayed contiguously for adenylate cyclase in a 10min assay at 24°C. The guanosine mono-, di- and tri-phosphates in combination with isoproterenol each produced a near 2-fold increase in activity over that produced by isoproterenol alone and were essentially equipotent (Table 1). Modification of the ribose moiety, for example (dGTP or 2'-O-methyl-GTP), caused a reduction in the activation relative to that produced by GTP. The absence of the phosphate group, as in guanosine, cyclization, as in cyclic GMP, or addition of a fourth 5'-phosphate, as in ppppG, all resulted in no activation. The purine base could be replaced by hypoxanthine, xanthine or cytosine but not uracil. Substitution in guanine at C-7 with a methyl group decreased the activity relative to that of the control. The $\alpha\beta$ - and the $\beta\gamma$ -phosphonate analogues of GTP produced the greatest degree of activation of adenylate cyclase; the $\beta\gamma$ -phosphoramidate analogue also activated the enzyme.

The concentration-dependence for nucleotide activation was examined by preincubation of membranes for 15min at 24°C with isoproterenol and nucleotide in the absence of the regenerating system followed by a 10min assay at 24°C (Fig. 1). These studies show that 0.01mM-GTP and -GDP produced near-optimal activation, whereas 1.0mM-GMP was required for optimal activation. The phosphate ester analogues also produced optimal activation at 0.01mM (Fig. 1a), and this was approx. 5-fold greater than basal activity.

Specificity for guanine nucleotide stabilization of adenylate cyclase

Previous studies showed cardiac adenylate cyclase to be unstable at 37°C, having a half-life of 4.4min, and p[NH]ppG produced stabilization of this activity (Snyder & Drummond, 1978). The activity is relatively stable at 24°C, the temperature of the assay. In the present studies, membranes were incubated for 15min at 37°C in the absence and presence

Fig. 1. Concentration-dependence for guanine nucleotide activation of adenylate cyclase

Membranes were incubated in the absence (○) or presence (closed symbols) of 0.1mM-isoproterenol with nucleotides as indicated for 15min at 24°C followed by 10min assay at 24°C in 10mM- Mg^{2+} /0.5mM- $[^3H]$ ATP.

of nucleotides (1.0mM), and then assayed at 24°C. Guanine nucleotides can also slowly activate adenylate cyclase in a time-dependent manner in the absence of hormone, and part of this activation involves a decrease in the amount of Mg^{2+} required for optimal activity (Snyder & Drummond, 1978). Assays were conducted in the presence of 25mM- Mg^{2+} in order to minimize any apparent activation. In the absence of any nucleotide, approx. 80% of adenylate cyclase activity was lost during the 15min incubation at 37°C (Table 2).

The phosphonate and phosphoramidate analogues of GTP were the most effective nucleotides in preventing a loss of activity; p[CH₂]ppG and pp[CH₂]pG actually produced a net increase in activity. GDP and GTP were more effective in stabilizing the activity than GMP. The triphosphates with modified ribose subsituents (dGTP and 2'-O-methyl-GTP) were less effective than GTP. Guanosine and UTP had no apparent effect and cyclic GMP and 7-methyl-GTP caused an apparent further destabilization of the activity. CTP, XTP and ITP had increasingly stabilizing effects (Table 2).

Examination of the concentration-dependent stabilization of adenylate cyclase by guanine nucleosides

Table 2. *Specificity of guanine nucleotide stabilization of cardiac adenylate cyclase*

Heart membranes were assayed at 24°C under standard conditions with 25 mM-MgCl₂ with or without a prior 15 min incubation at 37°C. In the absence of any additions and without preincubation, control activity was 105.2 ± 20.0 ($n = 10$) pmol/min per mg of protein, and with 15 min preincubation at 37°C activity was 18.9 ± 5.1 ($n = 10$) pmol/min per mg of protein. Results are expressed as means $\pm$ s.d. for the numbers of determinations given in parentheses and are activities relative to that in the absence of any additions or preincubation. Significance of difference from respective controls: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Additions (1.0mM)	Relative activity	
	No preincubation	Preincubation
None	1.00 ± 0.19 (10)	0.206 ± 0.017 (10)
(a) Modified phosphate		
Guanosine	1.13 ± 0.07 (3)	0.179 ± 0.030 (4)
Cyclic GMP	0.99 ± 0.04 (3)	0.154 ± 0.022 (4)
GMP	1.29 ± 0.10 (3)*	0.343 ± 0.048 (3)***
GDP	1.16 ± 0.12 (3)	0.719 ± 0.015 (3)***
GTP	1.01 ± 0.09 (4)	0.657 ± 0.045 (4)***
ppppG	0.68 ± 0.09 (3)*	0.444 ± 0.044 (3)***
ppGpp (MSI)	0.95 (1)	0.210 (1)
pppGpp (MSII)	0.91 (1)	0.239 (1)
p[NH]ppG	1.04 ± 0.08 (4)	1.082 ± 0.106 (4)***
p[CH ₂]ppG	0.69 ± 0.04 (3)*	2.362 ± 0.215 (4)***
pp[CH ₂]pG	0.75 ± 0.03 (3)*	2.105 ± 0.137 (3)***
(b) Modified ribose		
dGTP	0.98 ± 0.05 (3)	0.558 ± 0.016 (3)***
2'-O-Methyl-GTP	0.83 ± 0.06 (3)	0.419 ± 0.007 (3)***
(c) Modified base		
7-Methyl-GTP	0.82 ± 0.04 (4)	0.144 ± 0.019 (4)*
XTP	0.71 ± 0.07 (4)*	0.287 ± 0.016 (4)**
ITP	1.05 ± 0.04 (4)	0.529 ± 0.048 (3)**
UTP	0.99 ± 0.02 (3)	0.185 ± 0.010 (3)
CTP	1.06 ± 0.08 (3)	0.332 ± 0.015 (3)***

tides in the absence of a regenerating system showed GMP to have essentially no stabilizing effect, whereas 1.0mM-GDP and -GTP produced a nearly 3-fold stabilization compared with membranes in the absence of nucleotide (Fig. 2b). Stabilizing effects were evident at 0.001mM for the phosphate ester analogues of GTP, and they afforded maximal protection and greatest activity at approx. 1.0mM (Fig. 2b). There was no evidence of activation in the absence of the 37°C preincubation period, but the preincubation with p[CH₂]ppG or pp[CH₂]pG both stabilized and caused activation above basal levels.

Having observed a difference in the concentration of guanine nucleotide required to produce maximal activation and stabilization, the catabolism of GTP by membranes was examined at 24°C and at 37°C, the temperatures of the activation and stabilization studies respectively. After a 10min incubation of membranes with 1mM-GTP, 40% of the GTP remained at 24°C compared with only 12% at 37°C. Thus GTP was metabolized more rapidly under the conditions used for examining stabilization than under those used for activation studies, which may account for the difference in nucleotide concen-

trations required for maximal effect in these two situations.

Comparison of the structural requirements for activation and stabilization

By using the data obtained in these experiments, a comparison was made between the guanine nucleotide structural requirements for activation and stabilization of adenylate cyclase activity. As an index of nucleotide-dependent activation, the ratio of activities for membranes exposed to isoproterenol plus nucleotide to isoproterenol alone (derived from Table 1) was plotted against an index of stabilization, the ratio of activities for membranes preincubated at 37°C in the presence of nucleotide to those without nucleotide (derived from Table 2). A ratio of 1:1 indicates no apparent activation or no apparent stabilization relative to control values (Fig. 3). Nucleotides that had essentially no effect on either parameter were UTP, cyclic GMP, pppGpp and ppGpp. The phosphonate analogues of GTP, p[CH₂]ppG and pp[CH₂]pG, caused the greatest activation and stabilization of all the nucleotides

Fig. 2. Concentration-dependence for guanine nucleotide stabilization of adenylate cyclase

Membranes preincubated at 37°C for 15 min (closed symbols, solid line) or without preincubation (open symbols, broken line) were assayed at 24°C for 10 min in 25 mM-Mg²⁺/0.5 mM-[³H]ATP.

examined. At the other end of the spectrum, 7-methyl-GTP produced a decrease both in stability and activation of adenylate cyclase. A least-squares analysis of these data gave a correlation coefficient of 0.90 between the parameters of relative activation and stabilization.

Discussion

This study was designed to examine the nucleotide structural requirements for both hormone-facilitated guanine nucleotide activation and guanine nucleotide stabilization of adenylate cyclase. A model consistent with the previous (Snyder & Drummond, 1978) and present studies suggests that stabilization of cardiac membrane adenylate cyclase readily occurs upon exposure to guanine nucleotide, and activation is a slower time-dependent process which is accelerated by hormone. In other cells, reconstitution experiments have suggested that the guanine nucleotide-binding component of adenylate cyclase is thermostable and the catalytic unit is the thermolabile component (Ross *et al.*, 1978). Studies of adenylate cyclase activation and stabilization were therefore conducted with GTP analogues having modifications in the base, ribose and phosphate moieties. Any modification of the guanine base structure resulted

Fig. 3. Relationship between nucleotide activation and stabilization of adenylate cyclase

Relative activation was calculated from the data in Table 1 by setting the activity in the presence of isoproterenol alone equal to 1 (95 pmol/min per mg of protein) and expressing the activity of each compound plus isoproterenol relative to that with isoproterenol alone. Relative stabilization was calculated from the data in Table 2 by setting the activity of membranes preincubated for 15 min at 37°C equal to 1 (18.9 pmol/min per mg of protein) and expressing the activity for preincubation in the presence of each compound relative to preincubation without additions. Any nucleotide in the stippled area produced a decrease in both the activity and stability of adenylate cyclase. The line obtained by linear-regression analysis has a correlation coefficient of 0.90.

in a decreased effectiveness for stabilization. The absence of the 2-amino group, as in ITP, was the least critical change, and replacement by a 2-oxy group, as in XTP, produced a partial loss of stabilizing and activating potential. The pyrimidine UTP had no activating or stabilizing effect; however, CTP had significant stabilizing and activating ($P < 0.001$) properties. Substitution at N-7 with a methyl group produced a net decrease from control activities; the 7-methyl group eliminates the π -bonding interaction of N-7 and C-8 in the fused five-membered ring. In other studies of p[NH]ppG analogues, substitution at C-6 or N-2 reduced both the affinity for pigeon erythrocyte membranes and the ability to activate adenylate cyclase (Pfeuffer & Eckstein, 1976). In previous studies, preincubation of cardiac membranes at 37°C with ATP increased the lability of adenylate cyclase (Snyder & Drummond, 1978). In summary, of the nucleotides examined, the guanine base moiety provided the greatest degree of activation and stabilization. Modification of the ribose substituent, as in dGTP or 2'-O-methyl-GTP, also produced a decrease in both activation and stabilization relative to GTP.

Modification of the phosphate moiety produced the greatest variation in activation and stabilization. A requirement for a non-cyclized phosphate group was demonstrated by the absence of any effect caused by guanosine or cyclic GMP (Tables 1 and 2). GMP had no apparent stabilizing effect in the absence of a regenerating system (Fig. 2b), but did increase activity at a concentration of 1.0 mM in the presence of hormone (Fig. 1b). GDP was uniformly about 25% more effective than GTP in activating or stabilizing adenylate cyclase. The presence of a fourth 5'-phosphate, in ppppG, reduced the effectiveness relative to GTP, and addition of a 3'-diphosphate group in combination with the 5'-triphosphate (pppGpp) or 5'-diphosphate (ppGpp) essentially abolished any activation or stabilization. Modification of the terminal phosphate group of GTP in other studies by linkage to aromatic esters or amidates increased the binding to pigeon erythrocyte membranes and activation of adenylate cyclase (Pfeuffer & Eckstein, 1976). The most effective modifications were those affecting the phosphate ester linkage. Previous studies had shown the effectiveness of p[CH₂]ppG, pp[CH₂]pG and p[NH]ppG in activating adenylate cyclase (Salomon *et al.*, 1975; Spiegel *et al.*, 1977; Londos *et al.*, 1977). The phosphonates having a $\beta\gamma$ -methylene or $\alpha\beta$ -methylene bridge were the most effective activators and stabilizers of adenylate cyclase. The $\beta\gamma$ -imido bridge also produced marked stabilization; the activation was equivalent to that produced by GTP. The effectiveness of both pp[CH₂]pG and p[CH₂]ppG indicates that the guanine nucleotide site(s) need not possess either phosphatase or pyrophosphatase activities for

activation or stabilization of adenylate cyclase, although such activities could be responsible for reversing the nucleotide effects.

The present findings, taken together with known tissue concentrations of GTP (0.2–0.4 mM) and GDP (0.1–0.2 mM) (Deuticke & Gerlach, 1966), support a physiological role for guanine nucleotides in the cellular activation and stabilization of cardiac adenylate cyclase. The high degree of correlation in the structural requirements between the relative activation and stabilization of the various nucleotides examined (Fig. 3) is consistent with there being a common guanine nucleotide site mediating both these effects.

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