Catecholamine-Sensitive Adenylate Cyclase of Caudate Nucleus and Cerebral Cortex

EFFECTS OF GUANINE NUCLEOTIDES

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1. GTP and GMP-P(NH)P (guanylyl-5'-yl imidodiphosphate) were observed to increase the stimulation of neural adenylate cyclase by dopamine (3,4-dihydroxyphenethylamine) and noradrenaline.
2. GMP-P(NH)P had a biphasic effect on the enzyme activity.
3. Preincubation of membranes with GMP-P(NH)P activated the enzyme by a process dependent on time and temperature. Catecholamines increased the speed and the extent of this activation.
4. Membrane fractions contained high- and low-affinity sites for GMP-P(NH)P binding; this binding was due to protein(s) of the membrane preparations.
5. Low-affinity-site binding of GMP-P(NH)P appeared to be related to the stimulatory effect on the adenylate cyclase activity.

Several recent observations show that GTP plays an important, if not an essential, role in the regulation of hormonal responsiveness of adenylate cyclase (EC 4.6.1.1) in cell-free preparations derived from a variety of tissues. The nucleotide phosphohydrolase (EC 3.1.3.31)-resistant analogue of GTP, GMP-P(NH)P† (Yount *et al.*, 1971), which also stimulates adenylate cyclase, has proved useful in studies of mechanism(s) by which guanine nucleotides may regulate the catalytic reactivity of membrane-bound cyclase (see Rodbell *et al.*, 1975, and the references therein). The observed competitive interactions between GTP and GMP-P(NH)P in the stimulation of adenylate cyclase suggest that both interact at the same site(s) (Salomon *et al.*, 1975; Glossmann & Gips, 1974; Lefkowitz & Caron, 1975). However, only a limited number of preliminary studies has been carried out to elucidate the relationship between the binding of guanine nucleotides and the stimulation of the membrane-bound adenylate cyclase by guanine nucleotides (Glossmann & Gips, 1974; Glossmann, 1975; Salomon *et al.*, 1975; Jacobs & Cuatrecasas, 1976). Salomon *et al.* (1975) reported a relationship between the extent of activation of hepatic adenylate cyclase by GMP-P(NH)P and the amount of the nucleotide remaining bound to membranes after washing, and Jacobs & Cuatrecasas (1976) showed that

a considerable amount of GMP-P(NH)P is non-specifically bound to a membrane fraction and is unrelated to the action of this nucleotide on the enzyme. Here we describe the following observations: (i) dopamine (3,4-dihydroxyphenethylamine)- and noradrenaline-sensitivity of adenylate cyclase from neural tissues can be considerably increased by GTP and GMP-P(NH)P; (ii) GMP-P(NH)P showed a biphasic action; at lower concentration the cyclase activity was decreased (up to 30%), whereas at higher concentrations it was stimulated up to 3-fold; (iii) caudate-nucleus membrane preparations revealed multiple [³H]GMP-P(NH)P-binding sites; binding to the low-affinity site(s) appeared to be related to the effect on the cyclase activity. The properties of sites for GMP-P(NH)P binding are also described. Some of these findings have appeared in abstract form (Narayanan *et al.*, 1976).

Experimental

Materials

[α -³²P]ATP (15–18 Ci/mmol), cyclic [³H]AMP (38.4 Ci/mmol) and [8-³H]guanylyl-5'-yl imidodiphosphate were purchased from New England Nuclear, Montreal, Que., Canada. Nucleotides, nucleosides and bases were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., or P-L Biochemicals, Milwaukee, WI, U.S.A.; GMP-P(NH)P was from ICN Pharmaceuticals, Irvine, CA, U.S.A.; disposable columns were from Bio-Rad Laboratories, Richmond, CA, U.S.A.; creatine kinase and phospho-

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† Abbreviations: GMP-P(NH)P, guanylyl-5'-yl imidodiphosphate; GMP-P(CH₂)P, guanylyl-5'-yl methylene-diphosphonate; (Na⁺+K⁺)-ATPase, (Na⁺+K⁺)-dependent adenosine triphosphatase.

creatine were from Sigma. Distilled deionized water was used to wash all glassware and plasticware.

Methods

Preparation of homogenates and washed particles of rat caudate nucleus. Wistar rats (male, 200–300 g) were decapitated, their brains quickly removed and caudate nuclei (about 60 mg/rat) were separated. Caudate nuclei were homogenized with 10 vol. (v/w) of cold (4°C) 2 mM-Tris/maleate/2 mM-EGTA buffer, pH 7.4, by using a Potter–Elvehjem-type homogenizer (glass tube, Teflon pestle; 0.1–0.15 mm clearance) by hand (15 strokes). When used, homogenate was assayed within 30 min for adenylate cyclase activities. Washed particulate fraction was isolated by centrifuging the homogenate for 30 min at 40000g_{av.} in a Beckman JA-21 refrigerated centrifuge (rotor JA-20); the residue was washed (twice) by suspending it in cold (4°C) buffer (10 vol.) and centrifuging for 30 min at 40000g_{av.}. The residue was finally resuspended in cold buffer and assayed immediately for adenylate cyclase activities. The recovery of adenylate cyclase in washed particles averaged 85%.

Preparation of membrane fractions enriched in adenylate cyclase from rat and rabbit cerebral cortex and caudate nucleus. Animals were killed by decapitation, their brains quickly removed and cerebral cortices were separated. The tissue (about 3 g for cortex, 0.5 g for caudate nuclei) was then homogenized with 10 vol. (v/w) of cold (4°C) 10 mM-Tris/HCl/0.2 mM-dithiothreitol/10% (w/v) sucrose buffer, pH 7.8, with a Potter–Elvehjem homogenizer of construction and clearance as described above, by using a motor-driven pestle (300 rev./min, 15 strokes). This and all other operations were carried out at 4°C. The remaining procedure was essentially similar to that of Jones & Matus (1974). The gradient fraction (fraction 2), which was enriched in adenylate cyclase as well as ouabain-sensitive (Na⁺+K⁺)-ATPase activities, was used in the studies reported here; about 60% of total activities applied to the gradient were recovered in this band.

Adenylate cyclase assay. Fractions (50–100 µg) were incubated in the reaction mixture (0.15 ml) containing 50 mM-Tris/HCl (pH 7.5), 5 mM-MgCl₂, 8 mM-theophylline, 20 mM-phosphocreatine, 30 µg of creatine kinase, 1 mM-cyclic AMP and 0.8 mM-[α-³²P]ATP (20–40 c.p.m./pmol). Fractions were preincubated for 5 min at 30°C and the reaction was started by the addition of ATP. Other compounds, such as catecholamines, GTP, GMP-P(NH)P etc., were present during the preincubation period. Incubations were carried out for 5 min at 30°C and were terminated by boiling the tubes for 3 min. The remaining procedure was as previously described (Sulakhe *et al.*, 1976).

Other assays. Protein was determined by the

method of Lowry *et al.* (1951), P_i by the method of Taussky & Shorr (1953), and ATPase activity as described by Sulakhe *et al.* (1973).

Assay for [³H]GMP-P(NH)P binding. Membrane protein (50–80 µg) was preincubated at 30°C for 10 min in the reaction mixture (final volume 0.15 ml), containing 50 mM-Tris/HCl (pH 7.5), 5 mM-MgCl₂, 1 mM-cyclic AMP and 8 mM-theophylline. The reaction was started by the addition of [³H]GMP-P(NH)P (3000–5000 c.p.m./pmol). Samples (0.1 ml) were withdrawn at the desired times and filtered through Millipore discs (0.45 µm pore size); the discs were washed with 10 ml (2 × 5 ml) of ice-cold 50 mM-Tris/HCl (pH 7.5), dried and counted for radioactivity by liquid-scintillation spectrometry at a counting efficiency of 35%. Appropriate controls containing denatured membrane protein and no protein were run simultaneously. In the absence of protein, the radioactivity retained by the Millipore discs was negligible (about 1% of that in the presence of membrane protein).

Results

Basal adenylate cyclase activity of rat caudate-nuclei homogenates

In accord with previous observations (see Drummond & Ma, 1973, for review), rat caudate-nuclei homogenates contained high basal adenylate cyclase activities (Table 1). Addition of GMP-P(NH)P (0.1 µM) or EGTA (0.5 mM) decreased these activities; addition of both GMP-P(NH)P and EGTA produced about 55% inhibition of basal cyclase activity. The EGTA-induced decrease was partly lessened

Table 1. *Adenylate cyclase activity of rat caudate-nucleus homogenate*

Rat caudate-nucleus homogenate (50–80 µg) was incubated at 30°C for 5 min in the reaction mixture (0.15 ml) containing 50 mM-Tris/HCl (pH 7.5), 5 mM-MgCl₂, 8 mM-theophylline, 1 mM-cyclic AMP, 20 mM-phosphocreatine, 50 µg of creatine kinase and 0.7 mM-[α-³²P]ATP (30 c.p.m./pmol): when added, GMP-P(NH)P was at 0.1 µM and EGTA and CaCl₂ at 0.5 mM. Results are means ± S.E.M. of three experiments in which assays were carried out in duplicate. The numbers in parentheses represent percentage of control activity.

Additions	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)
None (control)	265 ± 6 (100)
+GMP-P(NH)P	154 ± 5 (58)
+EGTA	167 ± 2 (63)
+EGTA+CaCl ₂	202 ± 4 (76)
+GMP-P(NH)P+EGTA	117 ± 3 (44)
+GMP-P(NH)P+EGTA+CaCl ₂	158 ± 15 (60)

by the addition of an equimolar concentration of CaCl_2 ; under these conditions, the free Ca^{2+} concentration would be less than $1\text{ }\mu\text{M}$.

Biphasic effect of GMP-P(NH)P on adenylate cyclase

Lower concentrations of GMP-P(NH)P produced moderate inhibition, whereas higher concentrations of this nucleotide produced marked stimulation of adenylate cyclase activity in homogenate and membranes (Table 2). In our laboratory, this biphasic effect of GMP-P(NH)P was also observed in heart plasma membranes (N. Narayanan & P. V. Sulakhe, unpublished work).

Table 2. *Biphasic effect of GMP-P(NH)P on adenylate cyclase*

Caudate-nucleus homogenate and membranes were prepared and assayed for adenylate cyclase activity as described under 'Methods'. Results are means $\pm$ S.E.M. of three to five experiments.

Concn. of GMP-P(NH)P	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)	
	Homogenate	Membrane
0	165 $\pm$ 4	390 $\pm$ 10
0.1 nM	145 $\pm$ 4*	360 $\pm$ 8*
1 nM	138 $\pm$ 3*	320 $\pm$ 10*
10 nM	159 $\pm$ 5	350 $\pm$ 7*
100 nM	179 $\pm$ 4	425 $\pm$ 12
1 μM	194 $\pm$ 6†	500 $\pm$ 15†
10 μM	370 $\pm$ 14†	650 $\pm$ 18†
100 μM	410 $\pm$ 20†	730 $\pm$ 15†

* Activities are significantly lower ($P < 0.05$) than the control activity [zero GMP-P(NH)P] when analysed by Student's paired t test.

† Activities are significantly greater ($P < 0.05$) than the control activity.

Catecholamine sensitivity of adenylate cyclase of caudate-nucleus homogenate

Numerous studies have shown stimulatory actions of catecholamines on brain adenylate cyclase; however, the degree of stimulation was modest [see Drummond & Ma (1973) and Daly (1975) for reviews]. To determine whether guanine nucleotide(s) enhance the catecholamine-sensitivity, caudate-nucleus homogenates were incubated with either dopamine or noradrenaline in the presence of different concentrations of GMP-P(NH)P. As shown in Table 3, extra cyclic AMP synthesis caused by noradrenaline was increased in the presence of GMP-P(NH)P (1 and $10\text{ }\mu\text{M}$) and decreased in the presence of 10 nM -GMP-P(NH)P; $10\text{ }\mu\text{M}$ -GTP also decreased this extra synthesis at lower (1, 5, 10 and $25\text{ }\mu\text{M}$) noradrenaline concentrations. Basal activity was decreased moderately at lower (10 nM and $1\text{ }\mu\text{M}$) and increased at higher ($10\text{ }\mu\text{M}$) concentrations of GMP-P(NH)P; $10\text{ }\mu\text{M}$ -GTP increased the basal activity slightly. Dopamine-stimulated cyclic AMP synthesis was also further increased in the presence of $0.1\text{ }\mu\text{M}$ - and $1\text{ }\mu\text{M}$ -GMP-P(NH)P (Table 4); basal activity was, however, decreased at lower GMP-P(NH)P concentrations (1 nM). Whereas in the presence of $25\text{ }\mu\text{M}$ -GMP-P(NH)P basal activity was increased markedly, dopamine caused a decrease rather than an increase in the activity (Table 4). Also, $10\text{ }\mu\text{M}$ -GTP, although without significant effect on basal cyclase activity, increased the dopamine-dependent activity at various dopamine concentrations.

Adenylate cyclase in membrane fractions of cerebral cortex and caudate nucleus

In the absence of GMP-P(NH)P, rat cortex membrane-bound cyclase was not stimulated by either noradrenaline (Fig. 1) or dopamine (Fig. 2).

Table 3. *Effects of GTP and GMP-P(NH)P on adenylate cyclase activity of rat caudate-nucleus homogenate assayed in the absence and presence of various concentrations of noradrenaline*

Rat caudate-nucleus homogenates were prepared and assayed for adenylate cyclase activity as described under 'Methods'. Concentrations of noradrenaline, GTP and GMP-P(NH)P were as shown in the Table. Numbers in parentheses represent noradrenaline-dependent increase in the enzyme activity. Results are averages of two experiments assayed in duplicate; the duplicates agreed within 5%.

Noradrenaline (μM)	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)				
	Control	+GTP ($10\text{ }\mu\text{M}$)	+GMP-P(NH)P (10 nM)	+GMP-P(NH)P ($1\text{ }\mu\text{M}$)	+GMP-P(NH)P ($10\text{ }\mu\text{M}$)
0	195	205	180	165	285
1	207 (12)	205 (0)	180 (0)	201 (36)	354 (69)
5	222 (27)	205 (0)	180 (0)	207 (42)	373 (87)
10	237 (42)	213 (8)	180 (0)	230 (65)	391 (106)
25	258 (63)	247 (42)	191 (11)	249 (84)	415 (130)
50	265 (70)	273 (68)	210 (30)	258 (93)	435 (150)
100	269 (74)	285 (80)	222 (42)	264 (99)	426 (141)

Table 4. *Effects of GTP and GMP-P(NH)P on adenylate cyclase activity of rat caudate-nucleus homogenate assayed in the absence and presence of various concentrations of dopamine*

Rat caudate-nucleus homogenates were prepared and assayed for adenylate cyclase activity as described under 'Methods'. Concentrations of dopamine and of GTP and GMP-P(NH)P were as shown in the Table. Numbers in parentheses represent increase or decrease in the enzyme activity caused by dopamine. Results are averages of two experiments assayed in duplicate; the duplicates agreed with 5%.

Dopamine (μM)	Adenylate cyclase activity (pmol of cyclic AMP/min mg of protein)					
	Control	GMP-P(NH)P (1 nM)	GMP-P(NH)P (0.1 μM)	GMP-P(NH)P (1 μM)	GMP-P(NH)P (25 μM)	GTP (10 μM)
0	205	170	200	260	500	210
1	214 (9)	175 (5)	215 (15)	320 (60)	500 (0)	221 (11)
5	228 (23)	186 (16)	250 (50)	365 (105)	470 (-30)	244 (34)
10	236 (31)	200 (30)	266 (66)	396 (136)	456 (-44)	250 (40)
25	243 (38)	216 (46)	284 (84)	428 (168)	458 (-42)	270 (60)
50	247 (42)	194 (24)	285 (85)	464 (204)	460 (-40)	284 (74)

Fig. 1. *Noradrenaline stimulation of adenylate cyclase of rat cerebral-cortex membrane: effect of GMP-P(NH)P*
Rat cortex membranes (45 μg of protein) were incubated under standard assay conditions in the presence of various concentrations ($\circ$, none; $\blacksquare$, 10 nM; $\blacktriangle$, 1 μM ; $\bullet$, 10 μM) of GMP-P(NH)P and noradrenaline.

Fig. 2. *Dopamine stimulation of adenylate cyclase of rat cerebral-cortex membrane: effect of GMP-P(NH)P*
Rat cortex membranes (60 μg of protein) were incubated under standard assay conditions in the presence of various concentrations of dopamine and in the presence of ($\bullet$) 10 μM -, ($\blacktriangle$) 1 μM -, ($\blacksquare$) 10 nM-GMP-P(NH)P or in its absence ($\circ$).

However, in the presence of GMP-P(NH)P (1 and 10 μM), significant stimulation by catecholamine was observed. Rabbit cortex membrane-bound cyclase was, however, stimulated modestly by dopamine, and this stimulation was further enhanced in the presence of GMP-P(NH)P (10 nM and 1 μM); basal cyclase of these membranes was decreased at low (10 nM) and increased at high (1 μM) GMP-P(NH)P concentrations. Similar observations were made on membrane fractions of rabbit and rat caudate nucleus (results not shown).

Influence of time of incubation, pH, ATP and MgCl_2 on the stimulatory effect of GMP-P(NH)P

Adenylate cyclase activities (assayed with and without catecholamines) of caudate-nucleus and cerebral-cortex (either from rat or rabbit) homogenates and membrane fractions were linear with time up to 6 min of incubation at 30°C under the standard assay conditions (see under 'Methods'). In the presence of 10 μM -GMP-P(NH)P the linearity was maintained for a longer incubation time (e.g. up to 15 min). However, the stimulatory effect of GMP-P(NH)P on basal and catecholamine-stimulated activities was detectable as early as at 30 s of incubation (results not shown). The pH optimum for basal cyclase activity was rather broad (pH 7.5–9.0); in the presence of GMP-P(NH)P (with or without dopamine present), a slightly alkaline pH optimum (pH 8–9) was observed. At acidic pH (e.g. pH 5.5), the fall in activity was considerable (90%), especially when GMP-P(NH)P was present in the assays (results not shown). The affinity for ATP (0.1–0.2 mM) of the basal adenylate cyclase remained unaffected in the presence of catecholamines (1–100 μM), GMP-P(NH)P (0.5–10 μM) or both (results not shown). Therefore it appeared that these agents increased the V_{max} without any demonstrable effect on the K_m for ATP. Increasing MgCl_2 concentration in the assay increased the rate of adenylate cyclase,

Table 5. *Effects of guanine nucleotides on adenylate cyclase activity*

Homogenate, washed particles and membrane fractions were prepared and assayed for adenylate cyclase as described under 'Methods'. Other additions are as shown in the Table. When present, dopamine was 25 μM .

Addition (0.1 mM)	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)					
	Rat caudate nucleus				Rabbit cerebral-cortex membranes	
	Homogenate		Washed particles			
	-Dopamine	+Dopamine	-Dopamine	+Dopamine	-Dopamine	+Dopamine
None	128	178	178	232	228	512
GMP	129	181	195	261	441	706
GDP	181	177	141	152	285	594
GMP-P(CH ₂)P	453	384*	485	511	631	668
GTP	160	220	135	195	443	595
GMP-P(NH)P	510	455*	498	590	791	530*

* Decrease in activity caused by dopamine.

Table 6. *Effects of preincubation of membranes in the presence of dopamine, GMP-P(NH)P or both on adenylate cyclase activity assayed in the presence of variable dopamine concentrations and GMP-P(NH)P*

Rat caudate-nucleus particles (0.5 mg) were preincubated (23°C) in the reaction mixture (0.5 ml) containing 10 mM-Tris/maleate, pH 7.0, 0.2 mM-dithiothreitol, 0.5 mM-EGTA and, when present, 50 μM -dopamine and/or 25 μM -GMP-P(NH)P for 5 min. The suspensions were then centrifuged for 30 min at 40000 $g_{av.}$, and the pellet was washed by resuspension in 2 ml of cold buffer (2 mM-Tris/maleate, pH 7.0, 0.2 mM-dithiothreitol and 0.5 mM-EGTA) and centrifuging for 30 min at 40000 $g_{av.}$. The pellet was finally resuspended in 0.5 ml of cold (4°C) buffer and assayed for adenylate cyclase activity in the presence or absence of the concentrations of GMP-P(NH)P and/or dopamine indicated.

Additions to preincubation mixture		Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein)						
Dopamine (μM)	GMP-P(NH)P (μM)	GMP-P(NH)P (μM)	Additions to incubation mixture					
			Dopamine (μM)					
			0	1	5	10	25	50
—	—	—	104	124	123	101	102	103
		0.5	103	125	142	149	161	150
		10	237	307	325	336	329	329
		—	91	103	130	105	105	105
50	—	0.5	117	117	135	137	138	140
		10	260	321	352	392	387	360
		—	177	167	197	233	225	170
		0.5	192	183	215	238	221	200
—	25	10	336	435	498	461	443	415
		—	193	188	205	228	242	224
		0.5	238	247	250	257	266	265
		10	490	520	565	553	530	500

with half-maximal velocity occurring at 2–3 mM-MgCl₂. GMP-P(NH)P (10 μM), dopamine (25 μM) and noradrenaline (50 μM) did not appreciably influence the affinity for MgCl₂ (results not shown).

Effects of other guanine nucleotides

Among the nucleotides tested, GMP-P(NH)P and GMP-P(CH₂)P were most effective in stimulating basal enzyme activity in homogenate or membrane

fractions (Table 5). GDP and GTP, which stimulated adenylate cyclase in homogenate and membrane, inhibited the activity present in washed particles of caudate nucleus. GMP stimulated rabbit cortex membrane cyclase activity, although it was ineffective in other preparations. The dopamine-dependent increase in the cyclase activity was either abolished or decreased markedly by GDP in caudate-nucleus preparations. When the enzyme activity was maximally stimulated in the presence of GMP-

P(NH)P or GMP-P(CH₂)P, dopamine appeared to inhibit rather than increase the activity (also see Table 4). This is indicated by the asterisk in Table 5.

Effect of preincubation of membrane fraction with GMP-P(NH)P in the absence and presence of catecholamines

As shown in Table 6, basal enzyme activity was elevated by preincubation of membrane fraction with GMP-P(NH)P and with GMP-P(NH)P plus dopamine. During this procedure (preincubation followed by washing of membranes), dopamine stimulation of the enzyme was somewhat decreased. However, addition of GMP-P(NH)P in the adenylate cyclase assay mixture increased the dopamine-dependent activity. In other experiments, the activation of the enzyme by preincubation with GMP-P(NH)P showed both time- and temperature-dependence; dopamine and noradrenaline increased the rate and the extent of activation by GMP-P(NH)P. These findings are in general agreement with those reported by St. Louis & Sulakhe (1976) for the cardiac enzyme.

Characteristics of [³H]GMP-P(NH)P-binding sites in membrane fractions

To determine whether the binding of GMP-P(NH)P was involved in or related to the effect of this nucleotide on adenylate cyclase, the following studies were carried out. Qualitatively similar results were obtained with washed particles and membrane fractions of rat and rabbit caudate nucleus and cere-

bral cortex. Most (40–50%) binding of [³H]GMP-P(NH)P occurred within 2 min and reached a steady state by 30 min of incubation at 30°C. Binding was proportional to the protein concentration (up to 1 mg/ml) when assayed at different concentrations of GMP-P(NH)P (16 nM, 0.32 μM, 0.64 μM, 1.92 μM) (results not shown). About 80% of the total binding was observed to be associated with protein(s) of membrane preparations. Further, the nucleotide was not metabolized to any significant degree after its binding to membranes. When membrane preparations were incubated with various concentrations of [³H]GMP-P(NH)P, two binding sites, differing in their affinities, were resolved by the Eadie–Hofstee plot: Site I, with K_m 0.053 μM and V_{max} 61 pmol/10 min per mg of protein; Site II, with K_m 2.125 μM and V_{max} 170 pmol/10 min per mg of protein. Binding of the nucleotide to either site was temperature-dependent; increasing the temperature from 0 to 22°C increased the binding modestly, whereas there was insignificant further increase between 22 and 40°C (Table 7). The energy of activation, estimated by applying the Arrhenius equation, was 30.33 kJ/mol (0–22°C). Binding displayed a broad pH optimum, from pH 6.5 to 8.5 (Table 7). An involvement of a thiol group was evident from observations that *p*-chloro (or *p*-hydroxy)-mercuribenzoate decreased binding by 65% (half-maximal inhibition at approx. 20 μM of either) and that this was reversed by the addition of cysteine (Table 7). Binding was stable to storage at 4°C for up to 2 days and was somewhat increased (20–40%) by freezing the preparation at –20°C for 1 day (results not shown). Among a

Table 7. *Effect of temperature, pH and thiol-group blockers on [³H]GMP-P(NH)P binding by caudate-nucleus membrane fraction*

In Expt. 1, the incubation mixture contained 33 mM-Tris/maleate, pH 7.5, 1 mM-cyclic AMP, 5 mM-MgCl₂, 8 mM-theophylline, 0.32 μM-[³H]GMP-P(NH)P (3380 c.p.m./pmol) and 87 μg of protein in a total volume of 0.15 ml. In Expt. 2, the incubation mixture was as in Expt. 1 except that 33 mM-Tris/maleate of the indicated pH, 106 μg of protein and 0.64 μM-[³H]GMP-P(NH)P were present, and the temperature was 30°C. In Expt. 3, incubation conditions were as in Expt. 2 except that 33 mM-Tris/maleate, pH 7.5, was present. Abbreviations: pCMB, *p*-chloromercuribenzoate; pHMB, *p*-hydroxymmercuribenzoate. All results are average of duplicate incubations, and are expressed as pmol/10 min per mg of protein.

[³ H]GMP-P(NH)P binding					
Expt. 1		Expt. 2		Expt. 3	
Temperature (°C)	Binding	pH	Binding	Additions	Binding
0	16.7	5.5	59.0	None	58.1
5.5	21.2	6.0	66.4	1 μM-pCMB	53.9
9	28.1	6.5	68.7	1 μM-pHMB	52.3
15	32.5	7.0	68.8	10 μM-pCMB	34.5
22	33.4	7.5	68.6	10 μM-pHMB	34.9
27	33.6	8.0	69.5	50 μM-pCMB	20.6
31	40.2	8.5	65.6	50 μM-pHMB	19.8
39	41.8	9.0	63.0	50 μM-pCMB + 1 mM-cysteine	49.3
				50 μM-pHMB + 1 mM-cysteine	53.7

Fig. 3. Effect of GTP, GDP and unlabelled GMP-P(NH)P on the binding of $[^3\text{H}]\text{GMP-P(NH)P}$ to caudate-nucleus membranes

Incubations were carried out at 30°C in the standard assay mixture containing $0.50\text{ }\mu\text{M}$ - $[^3\text{H}]\text{GMP-P(NH)P}$, except that variable concentrations of GTP (Δ), GDP ($\blacktriangle$) and unlabelled GMP-P(NH)P ($\circ$) were present as shown. Other agents ($\bullet$) tested include GMP, guanosine, ATP, ADP, AMP, adenosine, noradrenaline, adrenaline and isoproterenol (all tested between 0.1 and $100\text{ }\mu\text{M}$). A 50% decrease in binding is marked by vertical lines.

variety of purine nucleotides, nucleosides and bases, as well as catecholamines, tested, only GTP, GDP and unlabelled GMP-P(NH)P were able to displace the bound $[^3\text{H}]\text{GMP-P(NH)P}$ (Fig. 3); half-maximal displacement was observed at $12.5\text{ }\mu\text{M}$ -GTP, $20\text{ }\mu\text{M}$ -GDP or $0.3\text{ }\mu\text{M}$ -GMP-P(NH)P. GMP-P(CH_2)P was equipotent with GMP-P(NH)P (results not shown). NaCl ($5\text{--}500\text{ mM}$) and KCl ($5\text{--}500\text{ mM}$) increased the binding moderately (50% increase at about 100 mM -NaCl or -KCl), whereas MgCl_2 ($0.1\text{--}50\text{ mM}$) inhibited the binding by 30%. In the latter case, 1 mM - MgCl_2 produced the maximal inhibition, which was not increased any further by increasing MgCl_2 up to 50 mM in the assay. CaCl_2 at lower concentration ($0.1\text{--}2\text{ mM}$) had no effect, but at higher concentration ($5\text{--}50\text{ mM}$) produced 10–30% inhibition of binding (results not shown). In other experiments, NaCl (188 mM) or KCl (150 mM) were observed to increase the affinity of the binding sites slightly without appreciably affecting the V_{max} of binding. Treatment of membranes with trypsin (ratios of membrane protein to trypsin of 10:1, 5:1, 2:1, w/w) caused a time- and concentration-dependent decrease (50–80%) in binding. The inhibitory effect of trypsin was blocked by the addition of trypsin inhibitor (results not shown).

Relationship between the binding and the effect of GMP-P(NH)P

Site I binding. When membrane preparations were treated with phospholipase C (membrane-protein/

phospholipase-C ratio of 20:1, w/w; treatment at 23°C for 10 min), the V_{max} of the binding to site I was unaffected, although the enzyme activity was significantly decreased (65–80%). Trypsin treatment (membrane-protein/trypsin ratio of 10:1, w/w; treatment at 23°C for 10 min), however, decreased binding to site I by 50% and caused complete inactivation of the enzyme. MgCl_2 abolished binding to site I and increased the enzyme activity (results not shown).

Site II binding. The extent of binding and the stimulation of the enzyme showed a parallel relationship [GMP-P(NH)P varied between 0.5 and $10\text{ }\mu\text{M}$]; the time course of binding and the enzyme stimulation, however, showed no such relationship. GTP displaced the binding and also inhibited the stimulation of the enzyme by GMP-P(NH)P. Treatment with either phospholipase C or trypsin caused a comparable decrease in the binding and the effect of GMP-P(NH)P on the enzyme (results not shown).

Discussion

We have observed that stimulation of neural adenylate cyclase by noradrenaline and dopamine is increased by GMP-P(NH)P. However, the effect of GMP-P(NH)P was biphasic. Interestingly, the stimulation of rat cerebral-cortex membrane-bound cyclase by catecholamine showed an absolute requirement for guanine nucleotide. Preincubation of membranes with GMP-P(NH)P resulted in the activation of the enzyme. This finding suggested that the effect on the enzyme was partially irreversible. Membrane preparations were observed to bind GMP-P(NH)P quite strongly and contained at least two binding sites of different affinities towards the nucleotide. The binding to the high-affinity site appeared unrelated to the effect on the enzyme, whereas binding to the low-affinity site may be related to the stimulatory effect on the enzyme. Perhaps it could be suggested that the coupling between the binding of nucleotide to site II (low-affinity site) and the effect on the enzyme requires thermal activation. For example, preincubation at 0°C with $10\text{ }\mu\text{M}$ -GMP-P(NH)P did not activate the enzyme, whereas at 30°C the activation was observed. However, at 0°C considerable binding of GMP-P(NH)P was evident. In other studies, we observed that the stimulatory effect of $5\text{ }\mu\text{M}$ -GMP-P(NH)P was not observed when adenylate cyclase assays were carried out at temperatures less than 25°C , whereas at $30\text{--}40^\circ\text{C}$ the stimulatory effect was very significant (3–5-fold). The precise relationship between binding and the action of GMP-P(NH)P on the enzyme, however, cannot be elucidated from the findings reported here. [Many membrane-bound enzymes and pro-

tein(s) may be able to bind GMP-P(NH)P.] For this purpose, a highly purified enzyme preparation would be required. Jacobs & Cuatrecasas (1976) reported that in turkey erythrocytes most of the [^3H]GMP-P(NH)P binding was not related to the effect on the enzyme. Some of the findings presented here support their view. The mechanism by which lower concentrations of GMP-P(NH)P decrease the cyclase activity is not clear.

Cuatrecasas *et al.* (1975) and Jacobs & Cuatrecasas (1976) suggested that pyrophosphorylation by GTP of the membrane proteins (including adenylate cyclase) may be involved in the regulation of the enzyme activity, whereas the findings of Pfeuffer & Helmreich (1975), Glossmann & Gips (1974) and Salomon *et al.* (1975) do not support this view. In our studies, most of the recovered GMP-P(NH)P, after its binding to membrane preparations, was not metabolized. However, this may not rule out the possibility that phosphorylation of the membrane protein(s) occurs, especially in the presence of GTP.

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