Ligand Requirements for the Relaxation of Adenylate Cyclase from Activated and Inhibited States†

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ABSTRACT: The turkey erythrocyte adenylate cyclase system binds tightly the inhibitory nucleotide GDP, and a pretreatment step with isoproterenol and GMP is required to restore activation. Under identical pretreatment conditions, the release of labeled nucleotide is complete within 1 min whereas the restoration of activation by Gpp(NH)p requires 15 min. A study of the ligand requirements of the slow step shows the following: (a) The role of GMP is that of an obligatory allosteric regulator. (b) Cholera toxin modification of the system abolishes the requirement for GMP with a considerable enhancement in the reaction rate. (c) GMP is without effect on the relaxation process with the activator Gpp(NH)p as the resident nucleotide. In sharp contrast, ethylenediaminetetraacetic acid (without effect in a GDP-occupied complex) markedly potentiates alterations from the Gpp(NH)p-occupied state. (d) Formation of a GDP/guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) hydbrid leads to the suppression of both F^- and Gpp(NH)p activation. F^- activation is restored by isoproterenol alone, while GMP is still required to restore Gpp(NH)p activation. The results suggest that covalent modification or nucleotide analogue occupancy of the regulatory complex can modify the allosteric role for GMP, with consequences for the rate of the slow step.

We have recently investigated the regulatory properties of the turkey erythrocyte adenylate cyclase system. Our initial interest in this system was prompted by the observation that its β-adrenergic receptor was not regulated by guanine nucleotides. We found that the reason for this lack of regulation of the β-adrenergic receptor was due to bound GDP at the regulatory complex. Clearance of bound nucleotide by using a pretreatment step with isoproterenol (Iso) and GMP led to the restoration of both receptor regulation and enzyme activation by guanine nucleotide (Lad et al., 1980a). Consistent with the clearance of bound GDP was a change in the β-adrenergic receptor from a low- to a high-affinity state for the agonist, isoproterenol. [It should be noted that the restoration in Gpp(NH)p activation was not due to the elimination of an inhibitory form of the regulatory component and that isoproterenol stabilization was also eliminated as an explanation (Lad et al., 1980a).] Subsequently, we utilized the pretreatment reaction to probe the organization of the multireceptor nature of this cyclase system (Lad et al., 1980b) and developed a complementation assay for the regulatory component (Lad et al., 1980c). Most recently, we have shown by target analysis that in this cyclase system the release of bound guanine nucleotide (by the use of the pretreatment step) does not alter the size of the complex (Nielsen et al., 1981; Rodbell et al., 1981). These studies utilized the pretreatment step (with Iso and GMP) but did not explore the characteristics of the pretreatment reaction itself.

In order to understand the nature of the pretreatment step in the activation process, we have addressed three questions pertinent to this reaction: (a) Does the rate of release of nucleotide occur at the same rate as restoration of activation by Gpp(NH)p? (b) Is a second nucleotide "GMP" obligatory in the pretreatment step, and is the requirement altered by covalent modification of the regulatory complex? (c) Does the type of nucleotide residing at the regulatory complex (activator or inhibitor) alter the rate or ligand (GMP) requirement of the pretreatment reaction? The results presented provide answers to these questions.

Experimental Procedures

Materials. GDPβS, Gpp(NH)p, [^{32}P]ATP, and [^{3}H]cAMP were purchased from ICN. ATP (type A-2382), cAMP, GTP, *dl*-isoproterenol, propranolol, GMP, GDP, and bovine serum albumin were obtained from Sigma Chemical Co. Cholera enterotoxin was purchased from Schwarz/Mann. All other chemicals were reagent grade.

Adenylate Cyclase Assay. Enzyme activity was assayed in a 100-μL final volume containing 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 20 μM cAMP, 5 mM creatine phosphate, 3.3 units of creatine phosphokinase, and 100 μM [^{32}P]ATP (100–500 cpm/pmol). The membrane protein was 15–30 μg/assay. Other effectors were present at the following concentrations: 10 μM Gpp(NH)p, 10 mM sodium fluoride, 10 μM isoproterenol, or 100 μM propranolol. Samples were incubated for 5 or 15 min at 30 °C. Cyclic AMP was isolated and measured by the method of Salomon et al. (1976).

Membrane Purification. The turkey erythrocyte plasma membrane was prepared exactly as described previously (Lad et al., 1980b) by the method of Cassel & Selinger (1978). The functional properties of the membrane are identical with those reported by other workers. The following features were observed in these membranes: (a) persistent inhibition of GDP; (b) distinct modes of activation and inhibition by adenosine; (c) an obligatory requirement for GMP in pretreatment; (d) a requirement for isoproterenol and GMP pretreatment prior to adherence of solubilized extracts to a GTP affinity column. The isoproterenol + Gpp(NH)p (I + G) activity was 32 (±8) pmol of cAMP min^{-1} mg^{-1} in 30 membrane preparations.

Pretreatment of the Membrane with Isoproterenol and Nucleotide (Lad et al., 1980a). Pretreatment of the turkey erythrocyte plasma membranes with 50 μM isoproterenol and 1 mM GMP was carried out at 30 °C for various periods of time as indicated in the figure legends. The incubation me-

† From the Kaiser-Permanente Regional Research Laboratory, Los Angeles, California 90027. *Received October 21, 1982; revised manuscript received February 2, 1983.* Supported by Grant 17 from the Department of Research and Education and in part by Biomedical Research Support Grant RR 05521-19 from the Kaiser Foundation Research Institute. Publication No. 70 of the Kaiser Regional Research Laboratory.

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0006-2960/83/0422-3278$01.50/0

dium included 10 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl, pH 7.5, and membrane protein at a concentration of 1 mg/mL. As a control for pretreatment, the membranes were incubated without added ligand, at either 0 or 30 °C, and no change in the activities was observed. The pretreatment was stopped by diluting the sample 35-fold into an ice-cold wash solution consisting of 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 250 mM sucrose (TDS). The membranes were centrifuged at 30000*g* for 12 min at 4 °C. The ratio of Gpp(NH)p/[isoproterenol + Gpp(NH)p] [G/(I + G)] activity was determined in an adenylate cyclase assay by incubating for 10 min at 30 °C. The wash was repeated twice. Cyclase activity was invariant to washing (Lad et al., 1980b). Carry-over of isoproterenol was carefully eliminated by the following methods: (a) The assay after pretreatment included propranolol. (b) The pattern of enhancement of Gpp(NH)p activation was independent of the volume of buffer used to remove bulk free ligand. (c) Washing the membranes after pretreatment caused no change in the G/(I + G) ratio. (d) The binding of [^{125}I]iodohydroxybenzylpindolol (I-HYP) to the pretreated membranes is equivalent to the binding in untreated membranes (Lad et al., 1980a). The ratio of propranolol + Gpp(NH)p to isoproterenol + Gpp(NH)p, (P + G)/(I + G), was also determined and was identical with the G/(I + G) ratio in the experiments. Thus, the changes in the ratio are due to alterations in guanine nucleotide interactions and not due to alterations in receptor occupancy. Treatment of the membranes with isoproterenol alone was without effect on any functional parameters. An isoproterenol-dependent stabilization is not involved (Lad et al., 1980b).

It should be emphasized that the duration of the adenylate cyclase assay in the measurement of the ratios of activities [G/(I + G), (P + G)/(I + G), basal/(I + G)] is not a factor in the results presented here. Assays of duration of 5 min gave results which were comparable to assays of up to 15 min. In addition, the ligand requirements were analyzed in a pretreatment reaction under simple and well-defined conditions prior to assay.

The results are expressed as the ratio of Gpp(NH)p/[isoproterenol + Gpp(NH)p] [G/(I + G)] activities. Within each experiment, the I + G activity is invariant with washing (Lad et al., 1980b). The value of the specific activity of I + G and the range of variation in this activity (±5%) are also provided. The specific activity with I + G is not sensitive to the length of preincubation or to the ligands present during such an incubation.

Direct Exchange of Guanine Nucleotides. The procedure was essentially as described by Cassel & Selinger (1978). Turkey erythrocyte membranes were preincubated in a buffer containing $MgCl_2$ (6 mM), ATP (0.3 mM), DTT (1 mM), 50 mM Tris, pH 7.5, and a regenerating system, 5 mM creatine phosphate and 3.3 units of creatine phosphokinase. The buffer included 0.25 μM [^{3}H]GTP (30000 cpm). The reaction was terminated by addition of unlabeled nucleotide (GTP) and propranolol to give concentrations of 100 and 20 μM, respectively. GDP as the unlabeled nucleotide gave similar results. The reaction mixture was cooled, and the membranes were washed 3 times by centrifugation (10000*g*, 10 min, 4 °C) and resuspended to 1 mg/mL in ice-cold buffer. The pellet was then incubated with nucleotide (GTP) at a concentration of 100 μM for 10 min at 37 °C. The membranes were washed to remove free nucleotide. By the convention utilized by others (Cassel & Selinger, 1978; Pike & Lefkowitz, 1981), the labeled nucleotide removed represents hormone-independent and therefore "nonspecific" binding. The catecholamine-induced release of the labeled nucleotide was initiated by the addition of 1 volume of membrane suspension to 4 volumes of buffer at 30 °C, in the presence of 1 mM GMP. Separate samples without hormone or with propranolol served as controls. The membranes were pelleted (>95% recovery activity). Two aliquots of supernatant (2.0 mL) were gently removed, and the released nucleotide was counted (three cycles) in duplicate and converted to dpm by using a quench correction. Correction for quenching is nearly identical for all samples. The absolute amount of nucleotide released is similar to that reported by Cassel & Selinger (1978).

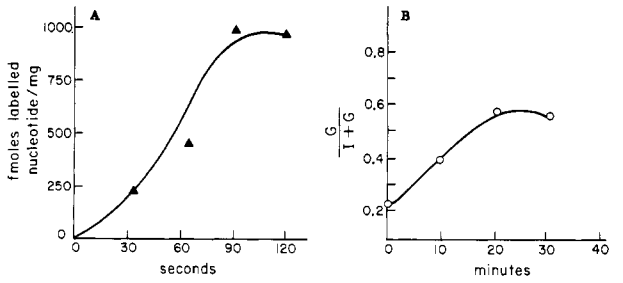

FIGURE 1: Comparison of the rates of direct exchange and restoration of activation. Membranes (1 mg/mL) were treated with isoproterenol (10 μM) and GTP (0.25 μM) with (▲) and without (○) label ([^{3}H]GTP, 30000 cpm), at 30 °C for 2 min, and washed in TDS to remove free nucleotide. The membranes were incubated with propranolol (30 °C, 2 min) to remove nonspecific nucleotide binding and washed with TDS. The final incubation was with isoproterenol and GMP; the parameter measured for direct exchange (▲) (A) is released of labeled nucleotide, while the second batch (○) (B) of membranes was assayed for restoration of Gpp(NH)p activation. The estimation of released nucleotide and the ratio of G/(I + G) are described under Experimental Procedures. The individual activities (G, I + G) at each time point were (8.6, 39), (16.3, 41), (23.2, 40), and (23.5, 41) pmol of cAMP min^{-1} mg^{-1}.

Cholera Toxin Treatment (Lad et al., 1980d). Cholera toxin (from Schwarz/Mann) was preactivated by incubation with 25 mM DTT at 30 °C for 15 min to generate the active fragment. The turkey erythrocyte membranes were treated with activated toxin (at a w/w ratio of 30:1), 30 °C, for 5 min with NAD (2 mM) and arginine (25 mM). The incubation was stopped by diluting the membranes 20-fold into an ice-cold wash solution consisting of 10 mM Tris-HCl, pH 7.5, 1 mM DTT, and 250 mM sucrose (TDS). The membranes were centrifuged at 30000*g* for 12 min at 4 °C and resuspended in ice-cold TDS. The degree of modification of the regulatory component was assessed by monitoring F^- activation of cyclase. Greater than 90% loss of fluoride activation was taken to be an index of complete toxin-catalyzed modification (Moss & Vaughn, 1979). Incubation of membranes under conditions identical with those used for toxin treatment, but in the absence of toxin, caused no significant change in any of the adenylate cyclase activities. The ratio G/(I + G) is identical with the ratio (P + G)/(I + G).

Results

Comparison of the Rate of Direct Release of Nucleotide with the Rate of Restoration of Activation by Gpp(NH)p. The results shown in Figure 1 indicate that in membranes treated identically with isoproterenol and GMP, the rate of direct release of nucleotide is rapid while the restoration of activation by Gpp(NH)p is slow (15 min).

It should be noted that an increase in the ratio of G/(I + G) is due only to an increase in Gpp(NH)p activity while the I + G activity ranged from 39.3 to 40.8 pmol of cAMP min^{-1} mg^{-1}. By contrast, the Gpp(NH)p activity increased from 8.6 to 23.5 pmol of cAMP min^{-1} mg^{-1}. Individual activities at each time point are given in the legend of Figure 1. In the ex-

Table I: Requirement for GMP in Restoration of Nucleotide Activation and Ligand Requirements for Relaxation from a GDP-Occupied State in Toxin-Treated Membranes

(A) GMP Requirements[a]		
x-fold dilution	ligand	(P + G)/(I + G)
1	Iso	0.07
5	Iso	0.07
10	Iso	0.08
50	Iso	0.07
1	Iso + GMP	0.40
5	Iso + GMP	0.32

(B) Ligand Requirements[b]	
ligand	ratio of (P + G)/(I + G) activities
none	0.05
GMP	0.09
Iso	0.70
Iso + GMP	0.92

[a] Turkey erythrocyte plasma membranes were pretreated by incubation for 30 min at 30 °C with isoproterenol (Iso) alone at different concentrations of the membrane. The original concentration was 20 μg/mL. The concentration of Iso was 10 μM in all incubations. GMP (1 mM) was present where indicated. The ratio of (P + G)/(I + G) was determined by assay of cyclase activity as described in the text. The I + G activity was 35 pmol of cAMP min^{-1} mg^{-1}, and the variation was within 5%. [b] Membranes were treated with cholera toxin and NAD as described under Experimental Procedures. The membranes were then incubated at 30 °C for 5 min in the presence of the appropriate ligands, washed, and assayed in the presence of propranolol + Gpp(NH)p and isoproterenol + Gpp(NH)p. The ratio of these activities is referred to as (P + G)/(I + G). The assay was for 15 min at 30 °C. The I + G activity was 37 pmol of cAMP min^{-1} mg^{-1} (±5%) for each of the determinations.

periments that follow, the I + G activity is stated and the range of variation noted. Thus, the relationship of the rate of release of nucleotide to the restoration of activation of Gpp(NH)p is complex. The requirements in direct exchange have already been explored in considerable detail (Cassel & Selinger, 1978; Cassel et al., 1977). The requirements for the slow restoration of activation are described below.

Role of a "Second" Nucleotide in Pretreatment. We have shown that the presence of GMP is required for the restoration of Gpp(NH)p activation of cyclase (Lad et al., 1980a). This requirement could represent either the prevention of rebinding of dissociated GDP or a regulatory role for a second site in nucleotide exchange. The experiment shown in Table IA was designed to distinguish between these possibilities. It was reasoned that if the rebinding of nucleotide (GDP) was the contributing factor in the lack of activation, then progressive dilution of the membrane (and hence of dissociated nucleotide) would yield activation in the presence of hormone alone. Membranes were diluted 1–50-fold in the presence of saturating isoproterenol without inclusion of GMP. The progressive dilutions did not cause any enhancement in activation. Inclusion of GMP at the lowest dilution gave a ratio of G/(I + G) activities of 0.32. These results suggest that there is an obligatory requirement for the presence of both hormone and a second nucleotide to effect activation and that the role for GMP is not one of merely preventing the rebinding of guanine nucleotide dissociated from the plasma membrane. The results obtained with the slow step are consistent with the results reported for direct exchange (Cassel & Selinger, 1978).

Modification by Cholera Toxin. An observation made frequently with allosteric enzymes is the alteration of site–site interactions after covalent modification. Cholera toxin (using NAD as substrate) catalyzes the ADP-ribosylation of the regulatory component. We examined the possible influence of toxin treatment on the GMP requirement in the pretreatment step. A time course of cholera toxin treatment was carried out, and pretreatment times corresponding to 30% (1 min) and 98% (5 min) loss of F^- activation were utilized. The toxin-treated membranes were then incubated with isoproterenol in the presence and absence of GMP, and the restoration of Gpp(NH)p activation was examined. As shown in Table IB, pretreatment with isoproterenol alone caused near-maximal restoration of Gpp(NH)p activation. Thus, in sharp contrast to the native membrane, GMP is no longer required for restoration of activation. This was further confirmed by carrying out the time course of pretreatment in toxin-modified and control membranes. In contrast to the native membrane where a 10-min incubation is required for restoration of activation, an incubation time of 1 min was sufficient for restoration of activation in toxin-treated membranes (Figure 2). Inclusion of GMP was without effect on the rate of restoration (data not shown) and had only a small effect on the half-maximal concentration for isoproterenol (insert of Figure 2). Thus, the ED_{50} for isoproterenol in the absence of GMP was 2×10^{-7} M and 0.6×10^{-7} M when GMP was present.

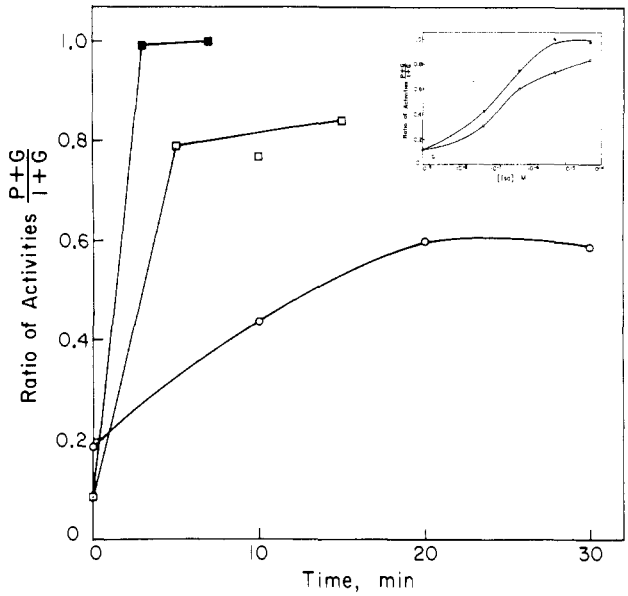

FIGURE 2: Restoration of Gpp(NH)p activation in control and toxin-treated membranes. Membranes were incubated without (○) and with cholera toxin and NAD for 1 (□) and 5 min (■), washed, and resuspended as described in the legend to Figure 1. The percent losses of F^- activity at these times of pretreatment were 30% and 98%, respectively. The membranes were then incubated with 50 μM isoproterenol and 1 mM GMP at 30 °C for various times. The incubation was terminated by the addition of 35 volumes of ice-cold TDS. The membranes were washed twice in the same buffer, resuspended, and assayed in the presence of propranolol (100 μM) and Gpp(NH)p (10 μM), or isoproterenol (10 μM) and Gpp(NH)p (10 μM); the assay conditions were 15 min, 30 °C. The inset shows the dose–response curve for isoproterenol in the presence (■) and absence (□) of GMP in the pretreatment of toxin-treated membranes (30 °C, 10 min). The I + G activity was 36 pmol of cAMP min^{-1} mg^{-1}, and the range of variation was <5%.

An obvious possibility to be explored was whether cholera toxin modification alters the persistent binding of GDP to the regulatory complex. This was tested by clearing the complex of GDP in both toxin-modified and native membranes, followed by incubation with GDP and an assay for inhibition of Gpp(NH)p activity. Persistent inhibition was observed in both native and toxin-modified membranes (Table II).

Ratio of Rates of Activation for G/(I + G). The value of G/(I + G) activities is increased from approximately 0.1 to 0.4–0.6 after treatment with isoproterenol and GMP. The

Table II: Persistent Binding of GDP in Toxin-Treated Membranes[a]

	first incubation, $F^- + P$ (pmol min^{-1} mg^{-1})	second incubation, ratio of (P + G)/(I + G) activities	third incubation, ratio of (P + G)/(I + G) activities
toxin-treated membranes	2.92	0.80	0.16
control membranes	23.80	0.61	0.15

[a] Membranes were incubated in either the presence or the absence of cholera toxin and NAD (first incubation) as described under Experimental Procedures. An aliquot of the membranes was retained to measure cyclase activity in the presence of propranolol (100 μM) and fluoride (10 mM). The rest of the membrane aliquot was incubated with GMP (1 mM) + isoproterenol (50 μM) at 30 °C for 20 min (second incubation). An aliquot of these membranes was retained to measure the ratio of (P + G)/(I + G) activity. The membranes were then incubated with (50 μM) GDP (third incubation), washed, and then assayed for the ratio of (P + G)/(I + G) activities at 30 °C for 15 min. The I + G activity was 30 pmol of cAMP min^{-1} mg^{-1}, and the variation was within 5%.

Table III: Comparison of the Conditions for Relaxation from Gpp(NH)p and GDP-Induced Activity States[a]

incubation 1		incubation 2		incubation 3	
ligand	G/(I + G)	ligand	G/(I + G)	ligand	G/(I + G)
Iso + GMP	0.43	Gpp(NH)p	1.0	Iso	0.41
				Iso + GMP	0.50
		GDP	0.17	Iso	0.18
				Iso + GMP	0.49

[a] Membranes were pretreated with isoproterenol (10 μM) and GMP (1 mM) (incubation 1, 30 °C, 20 min), and the ratio of activities in the presence of Gpp(NH)p and isoproterenol + Gpp(NH)p was measured. The membranes were then treated with Gpp(NH)p or GDP (incubation 2, 30 °C, 10 min), and the index G/(I + G) was measured in a 15-min assay. An aliquot was then washed to remove nucleotide and subjected to an incubation with isoproterenol alone or isoproterenol + GMP (incubation 3, 30 °C, 15 min). The membranes were again washed thoroughly, and the index G/(I + G) was measured as before. Adenylate cyclase assays were at 30 °C for 15 min. The ratio of basal/[isoproterenol + Gpp(NH)p] activity is also 0.1 after incubation 2 in the presence of Gpp(NH)p. The ratio of [propranolol + Gpp(NH)p]/[isoproterenol + Gpp(NH)p] [(P + G)/(I + G)] activity was determined and was identical with the G/(I + G) ratio, demonstrating that changes in the ratio are due to alterations in guanine nucleotide interactions and not receptor occupancy by isoproterenol. The I + G activity was 39 pmol min^{-1} mg^{-1} (±5%) for each determination.

index G/(I + G) is less than 1.0, suggesting differential access of Gpp(NH)p to the regulatory complex in the presence and absence of hormone. These observations raised the question of whether conditions could be devised so that the ratio G/(I + G) would be unity. An experimental approach toward this end is shown in Table III. Membranes were first treated with isoproterenol and GMP to clear the regulatory complex of GDP and then washed to remove free ligand. An aliquot of the membrane was assayed and G/(I + G) found to be 0.43. The membranes were then incubated with Gpp(NH)p to occupy the regulatory complex with this ligand and washed to remove bulk-free ligand. The pretreated membrane was assayed in the absence of any ligand ("basal"), in the presence of propranolol + Gpp(NH)p, and in the presence of isoproterenol + Gpp(NH)p. All three activities were equal. This sequence of incubations provides us with a means of obtaining

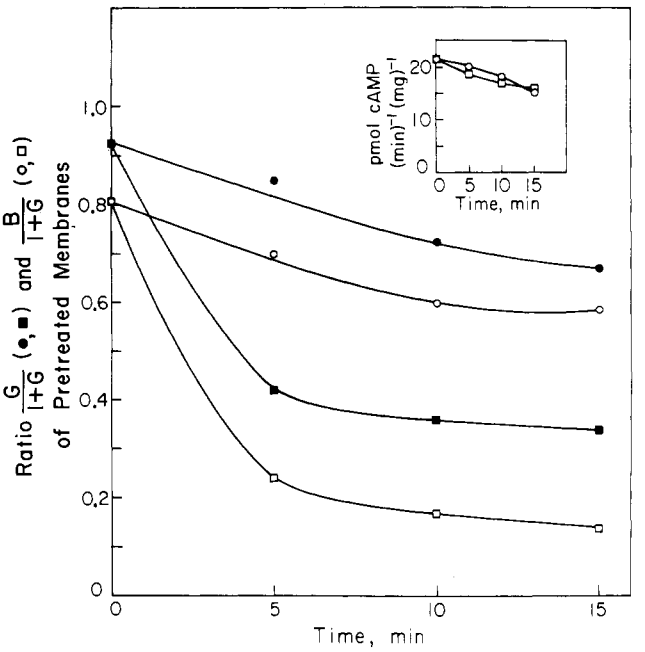

FIGURE 3: Relaxation from the Gpp(NH)p-induced state. Membranes (1 mg/mL) were incubated with isoproterenol (10 μM) + GMP (1 mM) and washed thoroughly to remove free ligands. The "GDP-cleared" membranes were then incubated (30 °C, 15 min) with Gpp(NH)p and washed to remove free nucleotide. The final incubation was with isoproterenol alone (10 μM; □, ■) or without ligand (○, ●) for the indicated times. The final incubation was stopped with cold TDS, and the ratio of G/(I + G) and B/(I + G) was determined as described under Experimental Procedures. The (P + G)/(I + G) ratio was identical with the G/(I + G) activity. The inset shows the specific activity in the presence of isoproterenol and Gpp(NH)p during the final incubation. The I + G activity was identical in preincubations carried out in the presence (□) or absence (○) of isoproterenol.

a maximal value of G/(I + G) activities of 1.0, a condition interpreted as equivalent to the complete occupancy of the regulatory complex by Gpp(NH)p. Subsequent incubation of these membranes with isoproterenol alone causes a decline in the ratio of activities from 1.0 to 0.4. Inclusion of GMP caused no significant alteration in the rate of change of G/(I + G) or in the final level of G/(I + G). In contrast to the nucleotide-independent mode observed with the relaxation from the Gpp(NH)p-occupied state, a different pattern was obtained with GDP as the resident nucleotide. The cycle of clearance of nucleotide [first incubation with isoproterenol + GMP] caused the expected increase in G/(I + G) from 0.18 (native membrane) to 0.43. Incubation of the membranes with GDP caused a diminution in the G/(I + G) activity to levels observed with the untreated membrane (0.17). When these sequentially treated membranes are exposed to isoproterenol alone, however, no further influence on the ratio G/(I + G) was observed. Inclusion of GMP in the third incubation caused an enhancement in G/(I + G) to levels observed in the first incubation (0.49). Thus, the ligand requirements for relaxation for Gpp(NH)p- and GDP-occupied states are clearly different. The Gpp(NH)p-occupied regulatory complex relaxes with isoproterenol alone, while the GDP-occupied complex shows a requirement for isoproterenol in conjunction with GMP. The relaxation from the Gpp(NH)p-induced state was also examined with isoproterenol and GDP. No effect of GDP was observed (data not shown).

The pattern of change in G/(I + G) in membranes sequentially treated with isoproterenol + GMP, followed by Gpp(NH)p, was investigated further in experiments described in Figure 3. Two ratios of activities are employed. The first is the ratio of G/(I + G) activities, and the second is the ratio

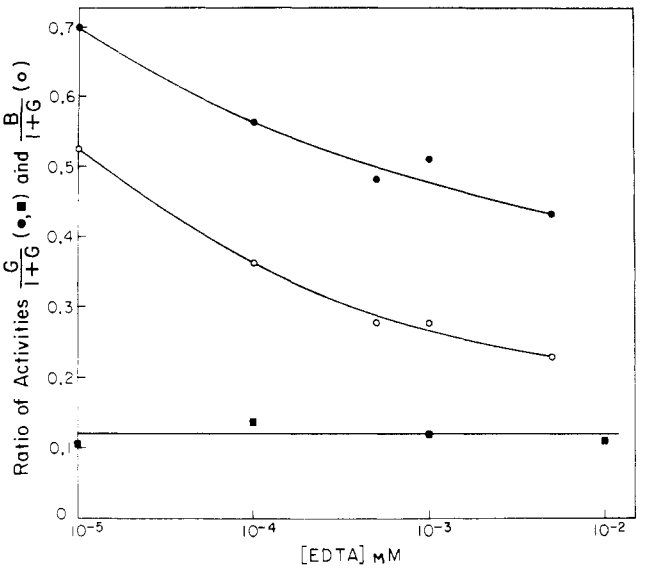

FIGURE 4: Influence of pretreatment with EDTA on subsequent nucleotide activation. Membranes (1 mg/mL) were treated sequentially with isoproterenol (10 μM) + GMP (1 mM) followed by Gpp(NH)p (10 μM) (○, ●) as described previously. The membranes were then incubated with varying concentrations of EDTA ± isoproterenol (10 μM) for up to 15 min at 30 °C, washed, and assayed in the presence of no ligand and in the presence of Gpp(NH)p. The ratios of activities of basal/[isoproterenol + Gpp(NH)p] (○) and of Gpp(NH)p/[isoproterenol + Gpp(NH)p] (●) were measured. Points designated (■) show the pattern obtained in incubations of the native (untreated) membrane with EDTA in the presence or absence of isoproterenol. Again, the (P + G/(I + G) ratio was equal to the ratio G/(I + G). The I + G activities at the various concentrations of EDTA (indicated in parentheses) were 38.3 (10^{-8} M), 39.8 (10^{-7} M), 36.7 (10^{-6} M), and 36.0 (10^{-5} M) pmol of cAMP min^{-1} mg^{-1}.

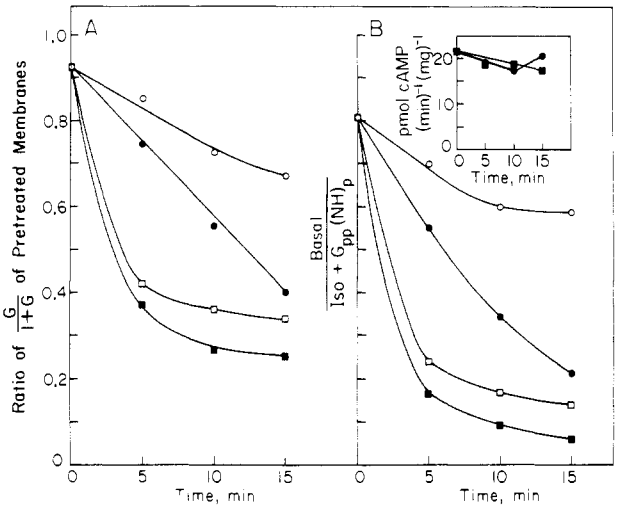

FIGURE 5: Effect of EDTA activities on the relaxation of the Gpp(NH)p-induced state. Membranes were treated with isoproterenol + GMP (1 mM) and then with Gpp(NH)p (10 μM) as described in the legend to Table II. These membranes were then incubated with isoproterenol (□, ■) in the absence and presence of EDTA (1 mM) at 30 °C for the times indicated. Symbols indicate incubation with no ligand (○) and with EDTA (●), Iso + EDTA (■). The membranes were then washed and resuspended with TDS (Experimental Procedures) and assayed in the presence of no ligand, Gpp(NH)p, and isoproterenol + Gpp(NH)p. The ratio of activities in the presence of Gpp(NH)p/[isoproterenol + Gpp(NH)p] (panel A) and of basal/[isoproterenol + Gpp(NH)p] (panel B) was determined. The inset represents the specific activities in the presence of isoproterenol + Gpp(NH)p during the preincubation with (■) or without (●) isoproterenol. The ratio (P + G)/(I + G) was equal to the ratio G/(I + G).

of basal (B) activity (activity measured without additions of ligand) to the isoproterenol + Gpp(NH)p activity [B/(I + G)]. Decline in the latter ratio has been shown to be proportional to the dissociation of bound Gpp(NH)p from the regulatory complex (Lad et al., 1980b). The decline is clearly not due to a change in the catalytic activity of adenylate cyclase (Figure 3 inset). Further incubation of the sequentially treated membranes caused a decrease in B/(I + G) to 0.6 (Figure 3). In the presence of isoproterenol, the dissociation of Gpp(NH)p was markedly enhanced as evidenced by a rapid change in the index of B/(I + G) from 0.8 to 0.1, indicative of enhanced dissociation of Gpp(NH)p from the regulatory complex, and in agreement with the observations of Cassel and Selinger (Cassel & Selinger, 1978; Cassel et al., 1977, 1979). Of most interest, however, is the decline of the ratio G/(I + G) activities in the absence of ligand to 0.7 and in the presence of isoproterenol to 0.4. If the site of dissociation of Gpp(NH)p remained freely accessible to Gpp(NH)p, then the ratio of G/(I + G) activities would be invariant. The results indicate that regardless of the mode of dissociation (spontaneous or hormone induced) of Gpp(NH)p, this nucleotide retains only partial access to the regulatory complex in the absence of hormone, after its dissociation.

Role of Ethylenediaminetetraacetic Acid (EDTA) in Relaxation Processes. EDTA has been shown by Cassel & Selinger (1978) to have an effect on the process of direct release of guanine nucleotide in the presence of isoproterenol. The influence of this agent on the relaxation of cyclase from activated and inhibited states was tested. The membranes were treated with isoproterenol + GMP [G/(I + G) = 0.43], washed, then incubated with either Gpp(NH)p [G/(I + G) = 1.0] or GDP [G/(I + G) = 0.18], washed to remove free ligand, and then incubated with EDTA alone or with isoproterenol and EDTA. To our surprise, no effect of EDTA was observed with GDP occupying the regulatory complex, and a substantial change in the ratio G/(I + G) (0.21) was observed when Gpp(NH)p was the resident nucleotide. This observation was further substantiated by the experiment shown in Figure 4. Treatment of the native membrane with isoproterenol and EDTA over a 1000-fold range in concentration of EDTA had no effect on G/(I + G). However, membranes which had undergone a sequential incubation with isoproterenol + GMP and then Gpp(NH)p showed a substantial effect with EDTA. A change was observed in parallel for the ratio of both basal/[isoproterenol + Gpp(NH)p] activity and Gpp(NH)p/[isoproterenol + Gpp(NH)p] activity. The half-maximal concentration for the effect of EDTA in these processes is 5×10^{-5} M. The time course of the EDTA-induced change in B/(I + G) and G/(I + G) is shown in Figure 5. While EDTA alone caused a rapid change in both ratios of activities, it did not alter the isoproterenol-induced relaxation from the Gpp(NH)p-induced state. Thus, after a 15-min incubation with isoproterenol, the ratio of activities G/(I + G) is 0.37. In the presence of isoproterenol and EDTA, the final level of G/(I + G) is somewhat lower (0.27). The process is complete within 15 min with hormone alone regardless of the presence or absence of EDTA. EDTA caused a shift in the ED_{50} for isoproterenol to lower concentrations from 10^{-7} to 2.5×10^{-8} M (data not shown). The effects of EDTA are observed only with Gpp(NH)p occupying the regulatory complex. Thus, nonspecific modulation is not involved in the results presented.

GMP Allosterism in GDPβS-Pretreated Membranes. Isoproterenol + GDPβS pretreatment of native membranes causes a loss of F^- activation of cyclase (Cassel et al., 1979). In these membranes, Gpp(NH)p and F^- activities are both lowered. A question of interest was whether the ligand re-

Table IV: Relaxation Modes of Adenylate Cyclase: Ligand Requirements and Times for Completion

state of membrane	resident nucleotide	parameter observed	ligand requirements	time for completion (min)
native	GDP	restoration of Gpp(NH)p activation	Iso and GMP[b]	20
pretreated[a]	GDP	restoration of Gpp(NH)p activation	Iso and GMP[b]	20
pretreated	Gpp(NH)p	decline of high activity state	(1) spontaneous (2) Iso (3) EDTA	<4
pretreated	GDP/GDPβS hybrid	(1) restoration of F^- activation (2) restoration of Gpp(NH)p activation	(1) Iso (2) Iso + GMP[b]	10
toxin treated	GDP	restoration of Gpp(NH)p activation	Iso	1

[a] Pretreated refers to the treatment of the membrane utilized to place the appropriate nucleotide within the regulatory complex. The cycle of pretreatment includes clearance of GDP with isoproterenol + GMP preincubation, followed by a nucleotide incubation. Native membrane refers to the untreated membrane. The assumption that GDP is the probable nucleotide at the regulatory complex in the native membrane has been supported by several recent studies (Cassel & Selinger, 1978). [b] GMP is obligatory in these steps.

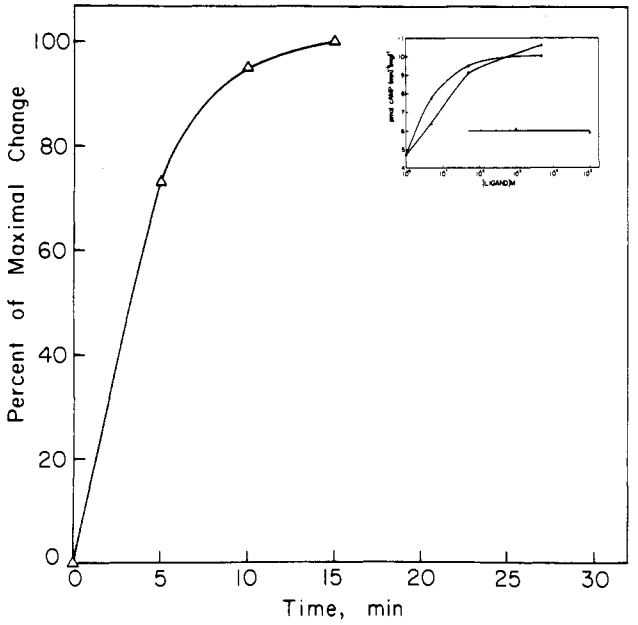

FIGURE 6: Time course of control and (isoproterenol + GDPβS)-treated membranes. Membranes were treated with 10 μM isoproterenol and 50 μM GDPβS (▲) at 30 °C for 10 min. Ice-cold TDS was added to stop the reaction. The membranes were centrifuged at 30000*g* for 15 min and then incubated with 50 μM isoproterenol for the times indicated. The incubations were stopped with ice-cold buffer, the membranes pelleted by centrifugation, and the membranes assayed with propranolol (100 μM) and F^- (10 mM) (△). The 100% activity was 11 pmol min^{-1} mg^{-1} (±5%). The inset shows the dose–response curve for isoproterenol in the presence (▲) and absence (△) of 1 mM GMP or GMP alone (○) in the treatment (30 °C, 10 min) of (Iso + GDPβS)-pretreated membranes.

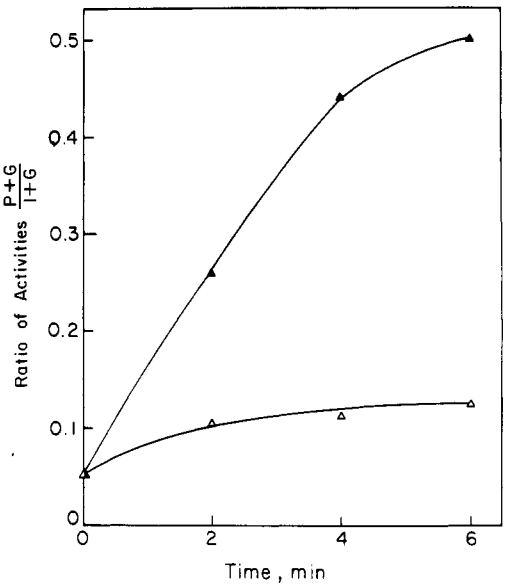

FIGURE 7: Restoration of Gpp(NH)p activation in GDPβS-pretreated membranes. Membranes were pretreated with 10 μM isoproterenol and 50 μM GDPβS under conditions described in the legend to Figure 1. The membranes were then incubated with 10 μM isoproterenol in the presence (▲) or absence (△) of 1 mM GMP, for 15 min at 30 °C, and washed, and the activities were measured in the presence of 10 μM Gpp(NH)p with 100 μM propranolol or 10 μM isoproterenol. The ratio of (P + G)/(I + G) activities was determined, as described in the legend to Table II. The I + G activity was 36 pmol min^{-1} mg^{-1} with a variation of ±5%.

quirements and rates for the restoration of F^- activation and Gpp(NH)p activation were similar. Experiments to test this possibility are described in Figures 6 and 7. When (isoproterenol + GDPβS)-pretreated membranes were treated with isoproterenol alone, F^- activation was restored, and the restoration of F^- activation was complete in 10 min. The ligand requirement for the restoration of F^- activation in isoproterenol + GDPβS membranes is shown in Figure 6. (Isoproterenol + GDPβS)-pretreated membranes were carried through a treatment with isoproterenol alone, GMP, and isoproterenol in the presence of GMP. No restoration of F^- activation was observed with GMP alone, while isoproterenol alone provided restoration of the F^- response (Figure 6). The ED_{50} values for isoproterenol in the presence and absence of GMP were 4.5×10^{-8} and 1.5×10^{-7} M, respectively. The rates of restoration of the F^- response in the presence of GMP and in its absence were also very similar (data not shown).

In contrast to the pattern observed with the return of F^- activation, when restoration of Gpp(NH)p activation was examined in (isoproterenol + GDPβS)-pretreated membranes, it was found that hormone alone could not restore Gpp(NH)p activation, and GMP was once again required to accomplish this process (Figure 7). The final ratio of activities [(P + G)/(I + G)] attained was the same as that observed for the untreated membrane (0.5).

Discussion

It is evident from our work (Lad et al., 1980a,b) and from the work of others (Pfeuffer & Helmreich, 1975) that pretreatment of turkey erythrocyte membranes with isoproterenol and GMP restores Gpp(NH)p activation, and the process is

compatible with the release of GDP from the regulatory complex. The results here provide further insights into two aspects of the reaction, the slow nature of the step and the ligand requirement for GMP. Thus, it is clear that the release of nucleotide from the complex is rapid while the restoration of activation is slow. These results are consistent with the results of Levitzki (1980), who has suggested that release of GDP is not rate limiting. In addition, they may be applicable to other cyclase systems, where slow kinetic phenomena are also observed. The studies by Neer & Salter (1981) on the brain adenylate cyclase system have suggested that transitions within regulatory proteins are important to the slow activation process.

As release of nucleotide is not the basis for the slow rate of the reaction, an explanation has to be sought elsewhere. The observation presented in this paper, namely, that cholera toxin treatment provides an acceleration in the rate of this process, is relevant. Abolition of the requirement for GMP is closely associated with the acceleration in rate. As GMP is an allosteric regulator of the reaction, the result implies that site–site interactions are of importance in the kinetic control of the reaction. Thus, our results provide a correlation between the ligand requirements and the rate of the reaction. It should be noted that the acceleration in rate is not due to the abolition of the tight binding of GDP. Rather, an alteration at the receptor/regulator interface is indicated.

Numerous studies [reviewed by Rodbell (1980)] have commented on the induction of a persistent high activity state induced by Gpp(NH)p in several cyclase systems. What is conspicuously absent, however, is a comparison of rates and ligand requirements for the relaxation from a GDP- vs. a GPP(NH)p-induced state. In particular, no information is available concerning the role of GMP in these processes. As it is known that the state induced by hormone + Gpp(NH)p is different from that induced by Gpp(NH)p alone, special care was taken to exclude the simultaneous presence of both ligands in the preincubation. This necessitated the use of an arduous three incubation sequence: clearance of GDP and occupancy by Gpp(NH)p followed by relaxation from the Gpp(NH)p-induced activity state. In view of the marked differences noted for the two relaxation processes, this caution seems to have been worthwhile. Two important differences have been noted. GMP which is obligatory with GDP at the regulatory complex is without effect under conditions of maximal occupancy of the regulatory complex by Gpp(NH)p. By contrast, EDTA is without effect in the GDP-occupied state but markedly potentiates the decline in the Gpp(NH)p-induced state. The type of nucleotide already resident at the regulatory complex determines the ligand requirements for relaxation as verified by our observations with the nucleotide GDPβS. In membranes treated with isoproterenol and GDPβS, both F^- and Gpp(NH)p activations are suppressed. We interpret this treatment as being indicative of the formation of a GDP/GDPβS hybrid. Removal of GDPβS from this complex (as assessed by restoration of the lost F^- response) does not require GMP. Further removal of GDP, however, still requires the combined presence of both isoproterenol and GMP, as would be expected from the native membrane. Our results are compatible with those of Hudson et al. (1981), who have shown that nucleotide analogues induce unique conformers of the regulatory complex.

The requirements for the relaxation of adenylate cyclase from various activated and inhibited states are summarized in Table IV. It is clear that the type of nucleotide residing at the complex and the state (native or modified) of the complex itself can affect both the ligand requirements and rates for these relaxation processes. Reconstitution studies (Ross & Gilman, 1980) are now in progress to define the role of individual subunits of the regulatory complex in each kinetic process.

Acknowledgments

We thank Dr. M. Michael Glovsky, Department of Allergy and Clinical Immunology, Southern California Permanente Medical Group/Kaiser Foundation Hospitals, and Dr. J. H. Richards, Department of Chemistry, California Institute of Technology, for their encouragement and support of this work. We acknowledge the help of Betty Aalseth in the preparation of the plasma membranes. We are indebted to Rosemary Castillo and Marijo Dowling for assistance in typing the manuscript.

Registry No. GDP, 146-91-8; GMP, 85-32-5; Gpp(NH)p, 34273-04-6; isoproterenol, 7683-59-2; EDTA, 60-00-4; GDPβS, 71376-97-1; adenylate cyclase, 9012-42-4.

References

Cassel, D., & Selinger, Z. (1978) *Proc. Natl. Acad. Sci. U.S.A. 45*, 4155–4159.

Cassel, D., Levkovitz, H., & Selinger, Z. (1977) *J. Cyclic Nucleotide Res. 3*, 393–406.

Cassel, D., Echstein, F., Lowe, M., & Selinger, Z. (1979) *J. Biol. Chem. 254*, 9835–9838.

Hudson, T. H., Roeber, J. F., & Johnson, G. L. (1981) *J. Biol. Chem. 256*, 1459–1465.

Lad, P. M., Nielsen, T. B., Londos, C., Preston, M. S., & Rodbell, M. (1980a) *J. Biol. Chem. 255*, 10841–10846.

Lad, P. M., Nielsen, T. B., Preston, M. S., & Rodbell, M. (1980b) *J. Biol. Chem. 255*, 988–995.

Lad, P. M., Nielsen, T. B., & Rodbell, M. (1980c) *FEBS Lett. 122*, 179–183.

Lad, P. M., Nielsen, T. B., Lin, M. C., Cooper, D. M. F., Preston, M. S., & Rodbell, M. (1980d) in *Novel ADP-Ribosylations of Regulatory Enzymes and Proteins* (Sugimura, T., & Smulson, M., Eds.) pp 202–205, Elsevier/North-Holland Press, New York.

Levitzki, A. (1980) *FEBS Lett. 115*, 9–10.

Moss, J., & Vaughn, M. (1979) *Annu. Rev. Biochem. 48*, 581–600.

Neer, E. J., & Salter, E. J. (1981) *J. Biol. Chem. 256*, 12102–12107.

Nielsen, T. B., Lad, P. M., Preston, M. S., Kempner, E., Schlegel, W., & Rodbell, M. (1981) *Proc. Natl. Acad. Sci U.S.A. 78*, 722–726.

Pfeuffer, T., & Helmreich, J. M. (1975) *J. Biol. Chem. 250*, 867–876.

Pike, L. J., & Lefkowitz, R. J. (1981) *J. Biol. Chem. 256*, 1459–1465.

Rodbell, M. (1980) *Nature (London) 284*, 17–22.

Rodbell, M., Lad, P. M., Nielsen, T. B., Cooper, D. M. F., Londos, C., & Preston, M. S. (1981) *Adv. Cyclic Nucleotide Res. 13*, 3–14.

Ross, E. M., & Gilman, A. G. (1980) *Annu. Rev. Biochem. 49*, 533–564.

Salomon, Y., Londos, C., & Rodbell, M. (1976) *Anal. Biochem. 58*, 541–548.