The interactions between the activatory guanine nucleotide binding protein and the catalytic subunit of adenylate cyclase in rat liver plasma membranes

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1. Three GTP-binding proteins of 50 kDa, 45 kDa and 28 kDa were identified by photoaffinity labelling with [γ - 32 P]GTP- γ -azidoanilide (A-GTP) in the rat liver plasma membrane. Pertussis toxin catalysed ADP-ribosylation of a single protein of 40 kDa. A-GTP had no effect on the basal labelling by pertussis toxin. After u.v. irradiation of the membrane in the presence of A-GTP, the GTP-dependent ADP-ribosylation by cholera toxin was increased, while the basal labelling was not affected. These results suggest that A-GTP interacts specifically with the activatory GTP-binding protein (G_s) and does not interact with the inhibitory GTP-binding protein (G_i). 2. The effects of partial photoinactivation of G_s of the rat liver plasma membrane adenylate cyclase system by A-GTP were studied. U.v. irradiation in the presence of increasing concentrations of the analogue caused progressive decrease in the maximal extent of activation by guanosine 5'-[γ -thio]triphosphate, but the K_a was not affected. 3. The rate of activation of liver adenylate cyclase by guanosine 5'-[γ -thio]triphosphate is temperature-dependent. The lag time increased from 0.5 min at 30 °C to 2.0–2.5 min at 15 °C in the presence of 10 μ M-guanosine 5'-[γ -thio]triphosphate. However, K_a remains unaffected by lowering the temperature. Photoinactivation by A-GTP or competitive inhibition by guanosine 5'-[β -thio]diphosphate decreases the maximal extent of activation by guanosine 5'-[γ -thio]triphosphate, but the lag time remains unaffected. 4. The present results support the idea that G_s is tightly associated with the catalytic subunit under basal conditions. The present results also indicate that the transition of an inactive G_s to its active form is the rate-limiting step of the activation of adenylate cyclase by guanosine 5'-[γ -thio]triphosphate in the intact rat liver plasma membranes.

INTRODUCTION

The activation and inhibition of adenylate cyclase by hormones requires at least three functionally distinct protein components, the hormone receptor (R), the catalytic unit (C) and two types of guanine nucleotide binding proteins, one mediating inhibition (G_i) and one activation (G_s) (Northup *et al.*, 1982; Sternweis *et al.*, 1981; Bokoch *et al.*, 1984; Codina *et al.*, 1984). Cholera toxin (CT) catalyses ADP-ribosylation of G_s in a reaction which requires GTP or its non-hydrolysable analogues (Cassel & Pfeuffer, 1978; Gill & Meren, 1978; Doberska *et al.*, 1980). *Bordetella pertussis* toxin (PT) ADP-ribosylates G_i (Katada & Ui, 1982).

In rat liver plasma membranes adenylate cyclase can be activated irreversibly by analogues of GTP such as GTP γ S or p[NH]ppG in the absence of hormones, in contrast with the turkey erythrocyte, where activation is critically dependent upon the presence of hormone (Lad *et al.*, 1977; Northup *et al.*, 1982; Sevilla *et al.*, 1976). As a result it is possible to investigate the mechanisms by which the catalytic unit is activated by G_s in rat liver plasma membranes, without taking account of the role of the hormone receptor.

The activation of adenylate cyclase by guanine nucleotides can be described as a three-stage process. First a guanine nucleotide binds to G_s , then G_s undergoes activation and finally G_s interacts with the catalytic unit

to increase catalytic activity. Recent studies have employed purified or partially purified preparations. The activity of G_s preparations has been accessed by reconstitution with cyc⁻S49 lymphoma cell membranes, which lack a functional G_s but contain a catalytic unit (Sternweis & Gilman, 1979; Iyengar, 1981; Asano *et al.*, 1984), or by reconstitution with purified preparations of catalytic unit (Cerione *et al.*, 1984). A problem with this type of approach is that it is difficult to determine the extent to which any effects observed have been influenced by the purification and reconstitution process.

In this study we have used the photoreactive GTP analogue GTP- γ -azidoanilide (A-GTP) to investigate the role of G_s in the activation of adenylate cyclase in intact rat liver plasma membranes. The analogue specifically and irreversibly inhibits the function of G_s (Wong & Martin, 1983) and can be used as a means of varying the amount of functional G_s in intact membranes. We have attempted to use this approach to determine which stage in the activation process is rate limiting.

EXPERIMENTAL

Materials

ATP, cyclic AMP, GTP, GTP γ S, phosphocreatine and creatine kinase were obtained from Boehringer (London), Lewes, East Sussex, U.K. Cyclic [3 H]AMP and [32 P]P_i

Abbreviations used: GTP γ S, guanosine 5'-[γ -thio]triphosphate; GDP β S, guanosine 5'-[β -thio]diphosphate; A-GTP, GTP- γ -azidoanilide, guanosine 5'-triphospho-5'-(4-azidoanilide); G_s , activatory GTP-binding protein; G_i , inhibitory GTP-binding protein; CT, cholera toxin; PT, pertussis toxin; C, catalytic unit of adenylate cyclase; p[NH]ppG, guanosine 5'-[β - γ -imido]triphosphate.

were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Cholera toxin was from Sigma. *Bordetella pertussis* toxin was supplied by Dr. L. Irons at the Public Health Laboratories Service, Porton Down, Salisbury, Wiltshire, U.K. [α - 32 P]ATP was prepared by the method of Martin & Voorheis (1977). [α - 32 P]NAD was synthesized from [α - 32 P]ATP as described by Cassel & Pfeuffer (1978). 4-Azidoaniline hydrochloride was obtained from Fluka and N^1 -(3-dimethylaminopropyl)- N -ethylcarbodi-imide hydrochloride from BDH. A-GTP and [γ - 32 P]A-GTP were prepared as described by Pfeuffer (1977) from 4-azidoaniline. The purity of A-GTP was checked by paper chromatography (Girshovich *et al.*, 1976) and t.l.c. on poly(ethyleneimine) plates (Pfeuffer, 1977).

Methods

Rat liver plasma membranes are prepared by the method of Pilgis *et al.* (1974).

Labelling with [α - 32 P]NAD and toxins. PT was preactivated by incubating 4 μ g/25 μ l lots in 50 mM-dithiothreitol for 1 h at 25 °C. CT was preactivated for 10 min at 37 °C in 20 mM-dithiothreitol. Activated toxins were used immediately. The labelling mix contained, in 100 μ l, 100 mM-potassium phosphate buffer, pH 7.0 (CT labelling) or 100 mM-Tris/HCl, pH 8.0 (PT labelling), together with 10 mM-thymidine, 1 mM-ATP, 50–75 μ g of membrane protein and 0.1–0.5 μ M-[α - 32 P]NAD (1×10^6 – 5×10^6 c.p.m.) which was added last to start the reaction. After 20 min at 30 °C, a stopping solution of 1 ml of ice-cold 50 mM-potassium phosphate buffer containing 0.1 mM-phenylmethanesulphonyl fluoride was added and membranes were washed once by centrifugation. Pellets were solubilized in 60 μ l of solubilizing buffer and proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis according to Laemmli (1970) using 10% acrylamide monomer. Autoradiography of dried gels using preflashed Fuji X-ray film was at –80 °C.

Adenylate cyclase activity. This was determined by the method of Salomon *et al.* (1974). Assay incubations were conducted at 30 °C for 15 min in a total volume of 0.1 ml containing 25 mM-Tris/HCl, pH 7.4, 10 mM-MgCl₂, 1 mM-dithiothreitol, 1.0 mM-cyclic AMP, 1 mM-EDTA, 0.5% bovine serum albumin, 0.5 mM-ATP, [α - 32 P]ATP (0.5 μ Ci/assay), 5 mM-phosphocreatine and 50 units of creatine kinase/ml. In some experiments, incubations were conducted at lower temperatures. In those circumstances more [α - 32 P]ATP (2–3 μ Ci/assay) and less unlabelled ATP (0.125–0.25 mM) were added to increase the specific radioactivities of the substrate and accordingly the sensitivity of the assay. Details are given in the legends.

Photoreaction with A-GTP. This was performed as described by Wong & Martin (1983) with the following modifications. Rat liver plasma membranes were incubated in the dark at 4 °C at a final concentration of 1 mg of protein/ml in the presence of A-GTP together with 25 mM-Tris/HCl, pH 7.4, 0.5% bovine serum albumin, 1 mM-EDTA, and 5 mM-ATP. Suspensions were irradiated at 4 °C at a distance of 5 cm with a Hanovia mercury lamp equipped with a cut-off filter at 366 nm.

Photoreaction was terminated by removing the sample from the u.v. source. At the end of the photoreaction, the membranes were washed four times at 4 °C with 50 vol. of buffer containing 25 mM-Tris/HCl, 0.25 mM-ATP, and 0.5% bovine serum albumin.

At the end of each wash, the pellet was dispersed mechanically by means of a syringe and the membrane

Fig. 1. Specificity of photolabelling by A-GTP in rat liver membranes

Lanes 1 and 2, photoaffinity labelling by [γ - 32 P]A-GTP. Liver membranes (60 μ g/track) was preincubated with 5 mg of bovine serum albumin/ml in 25 mM-Tris/HCl, pH 7.4, at 0 °C for 15 min, with (lane 1) or without (lane 2) GTP γ S. A mixture of [γ - 32 P]A-GTP (0.1 μ M) and ATP was then added. After standing for 10 min at 0 °C, the suspension was irradiated for 10 min at the same temperature. The final concentrations of albumin, ATP and GTP γ S during the irradiation were 2 mg/ml, 3 mM and 300 μ M respectively. The membranes were then washed and solubilized as described in the Experimental section. The radiographs of the dried gel are depicted above. Lanes 3 and 4, ADP-ribosylation by PT. Liver membranes (60 μ g/track) were ADP-ribosylated by PT as described in the Experimental section in the presence of 100 μ M-A-GTP (lane 3) and no additions (lane 4). The numbers (arbitrary units) presented at the bottom of each track represent the intensity of labelling, which was derived from the peak area after scanning the autoradiogram in a Joyce-Loebl densitometer.

pellet was finally resuspended in the washing buffer. Approx. 40 μg of membrane protein was present in each adenylate cyclase assay.

RESULTS

Specificity of A-GTP labelling

Fig. 1 compares the proteins which are photolabelled by [$\gamma\text{-}^{32}\text{P}$]A-GTP and ADP-ribosylated in the presence of

PT. Bands at 50, 45, 28 and 14 kDa were labelled by [$\gamma\text{-}^{32}\text{P}$]A-GTP. GTP γS specifically displaced labelling at the 50, 45 and 28 kDa bands (lanes 1 and 2). PT ADP-ribosylated a 40 kDa protein in the absence of any guanine nucleotide (lane 4). In contrast with GTP and GTP γS , which respectively enhanced and inhibited the basal ADP-ribosylation by PT (Wong *et al.*, 1985), A-GTP had no effect in the extent of labelling of the 40 kDa protein (lane 3).

Fig. 2(a) shows that after irradiating the membrane in

Fig. 2. Effects of A-GTP photolysis on ADP-ribosylation by CT

(a) Liver membranes (50 $\mu\text{g}/\text{track}$) were preincubated with 5 mg of bovine serum albumin/ml, 5 mM-ATP in 25 mM-Tris/HCl, pH 7.4, for 15 min at 0 °C. A-GTP (333 μM) was added and the suspension was allowed to stand for 10 min at 0 °C. The membrane suspension (1.67 mg/ml) was divided into two lots. One was subjected to u.v. irradiation for 1 h (lanes 1 and 3) while the other lot remained in the dark (lanes 2 and 4). After extensive washing of the membranes with ice-cold 25 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol, CT labelling experiments were performed without (lanes 1 and 2) or with lanes (3 and 4) 50 μM -GTP as described in the Experimental section. (b) Absorbance scan for autoradiograms of (a). The autoradiograms shown in (a) were scanned on a Joyce-Loebl microdensitometer. Lanes A, B, C and D respectively represent the scanning trace for lanes 3, 4, 1 and 2 in (a). The intensity of labelling at the 44 kDa band in A, B, C and D are 23.5, 15.0, 11.5 and 10.5 (arbitrary units) respectively.

Fig. 3. Effect of A-GTP photoinactivation on the activation by GTP γ S of liver membrane adenylate cyclase

Various amounts of A-GTP (as indicated in the Figure) were added to liver membrane (30 μ g/assay), which was subjected to u.v. irradiation for 30 min at 4 °C in the presence of 3 mM-ATP, 0.4% bovine serum albumin and 20 mM-Tris/HCl, pH 7.4, as described in the Experimental section. After extensive washing in 0.5 mM-ATP/1.25 mM-dithiothreitol/15 mM-Tris/HCl (pH 7.4)/0.3% albumin, adenylate cyclase assays were performed in the presence of 1.2 mM-ATP and a range of GTP γ S concentrations as indicated. Individual data are means $\pm$ S.E.M. for three separate incubations and are within 5% of the mean value. The stimulation (-fold) over basal activities is presented.

the presence of A-GTP and extensive washing, the GTP-dependent ADP-ribosylation by CT of the 50 and 44 kDa proteins was increased (lanes 3 and 4), while labelling in the absence of guanine nucleotide was not affected (lanes 1 and 2). Fig. 2(b) compares the extent of labelling more quantitatively. In the presence of GTP, the extent of labelling of the A-GTP-photolysed membrane was enhanced by 50%, while the basal labelling was not affected. These results suggest that A-GTP specifically interacts with G_s and does not bind to G_i (see also the Discussion).

The lack of interaction between A-GTP and G_i has also been shown in the human platelet membranes. In the dark, the photosensitive GTP analogue activated basal adenylate cyclase activity, but did not support adrenaline inhibition in the presence of forskolin. Moreover, GTP-mediated adrenaline inhibition remained intact after A-GTP photolysis, while the activation by 10 mM-NaF was reduced by 50% (S. K. F. Wong, unpublished work). Photoaffinity labelling of G_i by 8-azido-[γ -³²P]GTP has been reported (Bokoch *et al.*, 1983). The difference in the ability to label G_i between these two photoreactive analogues of GTP suggests that the bulky group on the γ -phosphate of the A-GTP hinders the binding of the guanine nucleotide analogue to G_i.

Having established the specificity of the A-GTP labelling, we further studied the effects of A-GTP photolysis on the activation of adenylate cyclase by GTP γ S.

Fig. 4. Effect of thermal inactivation on activation of adenylate cyclase by GTP γ S

Liver membranes (25 μ g/assay) ($\square$) were incubated at 30 °C for 20 min in the presence of 25 mM-Tris/HCl, pH 7.4, containing 1.25 mM-dithiothreitol, 5 mM-EDTA and 0.5% bovine serum albumin. Membranes were then kept in ice until adenylate cyclase assays, which were performed in the presence of 0.5 mM-ATP, using a range of GTP γ S concentrations as indicated. Control membranes ($\circ$) have not been preincubated at 30 °C. The stimulation (-fold) by GTP γ S over basal activities is presented.

Effects of A-GTP photoinactivation on the activation of adenylate cyclase by GTP γ S

Fig. 3 shows that increasing the concentration of A-GTP during photoreaction led to a progressive decrease in the extent of activation by GTP γ S. However, the K_a for GTP γ S determined from a double-reciprocal plot was unchanged at 1.6×10^{-8} M, as was the basal activity in the absence of GTP γ S. The possibility that A-GTP might be acting upon the catalytic unit was considered. The catalytic unit is more sensitive to thermal inactivation than is G_s (Iyengar & Birnbaumer, 1981) and this may be used as a means of selectively inactivating C. Fig. 4 shows the effect of thermal inactivation on the response of adenylate cyclase to GTP γ S. There was no effect on the K_a of activation by GTP γ S but, in contrast with the effects of photoreaction with A-GTP, basal activity was reduced to the same extent as GTP γ S-stimulated activity. It appears therefore that photoreaction in the presence of A-GTP does not affect the catalytic unit, which supports our earlier conclusions (Wong & Martin, 1983) (see also Fig. 7a). The K_a for GTP γ S was not affected by inactivation of the catalytic unit.

Effect of variations in temperature on the activation of adenylate cyclase by GTP γ S

The activation of adenylate cyclase by GTP γ S displays a lag phase which at 30 °C lasts for 30 s. Reducing the temperature from 30 °C to 15 °C leads to an increase in the length of the lag phase to 2–2.5 min (Fig. 5). The extent of activation was also reduced from 9-fold at 30 °C to 3.5-fold at 15 °C, but the reduction in temperature had little effect on the K_a for activation by GTP γ S, which

Fig. 5. Effect of temperature on the rate of GTP γ S activation of adenylate cyclase

Liver membranes (37 μ g/assay) were added to the adenylate cyclase assay mixture as described in the Experimental section containing 1 mM-EDTA, 10 mM-Mg $^{2+}$, 0.23 mM-ATP and 10 μ M-GTP γ S. After incubation for varying times at 30 °C (○) and 15 °C (□), 100 μ l aliquots were withdrawn, the reaction was stopped and the cyclic [33 P]AMP formed was estimated as described in the Experimental section. At both 30 °C and 15 °C, cyclic AMP accumulation curves were linear in the absence of GTP γ S; stimulations due to the addition of GTP γ S were 9- and 3.5-fold at 30 °C and 15 °C respectively.

Fig. 6. Activation of adenylate cyclase by GTP γ S at 15 °C

Liver membranes (30.3 μ g/assay) were incubated with varying concentrations of GTP γ S in the adenylate cyclase assay at 15 °C (○) and 30 °C (▲) in the presence of 0.125 mM-ATP and 10 mM-Mg $^{2+}$ as described in the Experimental section. The percentage of maximal stimulation (adenylate cyclase activity at 10 $^{-4}$ M-GTP γ S) is shown.

Fig. 7. Effect of A-GTP photoinactivation on the activation of adenylate cyclase by GTP γ S

(a) Liver membranes (37.5 μ g/assay) were preincubated with the indicated amount of A-GTP at 4 °C in the presence of 5 mM-ATP, 0.5% bovine serum albumin, 25 mM-Tris/HCl, pH 7.4, 1 mM-EDTA and subjected to 10 min u.v. irradiation as described in the Experimental section. After extensive washing with 0.25 mM-ATP/1 mM-EDTA/0.5% albumin in 25 mM-Tris/HCl, pH 7.4, adenylate cyclase assays were performed at 30 °C using 10 μ M-GTP γ S (○). Basal activity is expressed as (□), with water as control. (b) Liver membranes (37.5 μ g/assay) were treated essentially as in (a). After 10 min irradiation with (○) or without (△) 500 μ M-A-GTP and subsequent washings, the membranes were added to the adenylate cyclase assay mixture containing 1 mM-EDTA, 10 mM-Mg $^{2+}$, 0.25 mM-ATP and 10 μ M-GTP γ S at 15 °C. Kinetic experiments were performed as described in the legend to Fig. 5, except that the temperature of incubation was 15 °C.

Fig. 8. Effects of GDP β S on the kinetics of adenylate cyclase activation by GTP γ S

Liver membranes (48 μ g/assay) were incubated with the adenylate cyclase assay mixture with 0.125 mM-ATP and 10 μ M-GTP γ S at 15 °C, in the presence of 0 (Δ), 10 ($\square$) and 100 ($\circ$) μ M of GDP β S. Kinetic experiments were performed as described in the legend of Fig. 7(b).

Table 1. Effect of photoinactivation of A-GTP and GDP β S on the rate of adenylate cyclase activation by GTP γ S

The apparent first-order rate constant (τ) was calculated from Figs. 7(b) and 8 according to Neet & Ainslie (1980). The experimental conditions for experiments 1 and 2 are listed in the legends of Figs. 7(b) and 8 respectively.

Expt. 1		Expt. 2	
[A-GTP] (μ M)	τ	[GDP β S] (μ M)	τ
0	1.35	0	2.39
500	1.36	10	2.58
		100	2.39

was 2.6×10^{-8} M at 30 °C and 1.5×10^{-8} M at 15 °C (Fig. 6).

The reduction in the rate of activation by GTP γ S at low temperatures makes it easier to follow the kinetics. Figs. 7 and 8 show the effects of GDP β S or photoreaction with A-GTP on the kinetics of activation by GTP γ S at 15 °C. The extent of activation by GTP γ S was decreased without affecting the basal activity (Fig. 7a). The length of the lag phase before a linear rate was achieved was also unchanged (Fig. 7b). Similar results were obtained using the GDP analogue GDP β S, which inhibits the activation of adenylate cyclase by GTP or its non-hydrolysable analogues through competitive inhibition (Eckstein *et al.*, 1979). Increasing concentrations of GDP β S led to a reduction in the extent of activation by GTP γ S but did

not affect the length of the lag period (Fig. 8). The apparent first-order rate constants for the activation were calculated from the data in Figs. 7 and 8 according to Neet & Ainslie (1980) and are shown in Table 1. Neither reduction of the number of functional G_s units nor inhibition of G_s by GDP β S had any effect on the rate constant of activation.

DISCUSSION

In this study, we have established the specificity of the interaction between A-GTP and G_s . We were able to show that A-GTP specifically labelled three membrane-bound proteins of 50, 45 and 28 kDa and did not label the PT substrate G_i (40 kDa) or G_o (39 kDa) which has been reported recently (Sternweis & Robishaw, 1984). In the dark, A-GTP did not affect the basal ADP-ribosylation by PT. Since any guanine nucleotide which binds to G_i increases or decreases labelling by PT (Bokoch *et al.*, 1984; Katada *et al.*, 1984; Wong *et al.*, 1985), this suggests that A-GTP does not interact with or has a very low affinity for G_i .

The specificity of the interaction between A-GTP and G_s is also demonstrated by the observation that, in the dark, A-GTP activated rat liver membrane adenylate cyclase (Wong & Martin, 1983) and supported ADP-ribosylation by CT (Wong *et al.*, 1985) to similar extent as GTP. Since the ability of A-GTP to activate adenylate cyclase was found to be similar to its ability to stimulate CT labelling, one would expect a decrease in CT labelling upon A-GTP photolysis. However, under conditions in which at least 50% of G_s was inactivated by A-GTP photolysis (Fig. 3), the ability of GTP to catalyse CT labelling was enhanced by 50% while the basal CT labelling was not affected (Fig. 2). It would appear that covalent modification by A-GTP photolysis did not result in the permanent occupancy of the GTP binding site which mediates ADP-ribosylation, whereas it did permanently modify G_s to a form which was more susceptible to labelling by CT in the presence of GTP. Gill & Meren (1983) have reported the existence of distinct nucleotide-binding sites mediating adenylate cyclase activation (N site) and CT labelling (S site). It is possible that A-GTP photolysis specifically attacks the 'N' site, while leaving the 'S' site relatively intact. This may increase the binding of GTP to the 'S' site indirectly. The stability of G_s towards treatment with *N*-ethylmaleimide (Ross *et al.*, 1978; Wong *et al.*, 1985) is another indication that the 'S' site is relatively stable towards electrophilic attack.

The activation of adenylate cyclase by non-hydrolysable analogues of GTP such as GTP γ S can be described by the following scheme:

where G_s and C represent the basal states and G_s^* and C^* the activated states of G_s and C respectively. The above pathway represents only the reaction sequence and makes no prediction regarding the physical association between G_s and C, and two models have been proposed. In the first, G_s and C are tightly associated under basal conditions (Tolkovsky *et al.*, 1982). In the second model, G_s and C are separated under basal conditions and their association is a consequence of the activation of G_s to G_s^* .

(Citri & Schramm, 1980; DeLean *et al.*, 1980). It follows from the second model that free G_s can exchange readily with G_s bound as part of a $G_s \cdot C$ complex and there is no need to assume equal concentrations of functional G_s and C. The first model assumes that from the point of view of functions the $G_s \cdot C$ complex operates as a single unit. If there is an excess of G_s over C the rate of exchange of free G_s with G_s complexed with C is very slow relative to the activation process.

We have been using A-GTP to inactivate partially G_s to test the above models. According to the second model, partial inactivation of G_s would lead to its replacement by intact G_s remaining to interact with C. As more GTP γ S would be required to achieve this new equilibrium, the K_a would be expected to shift to a higher value. On the other hand, the first model would predict that a reduction in the concentration of G_s would lead to a reduction in the extent of activation by GTP γ S but no change in K_a , as G_s and C are closely associated as a single $G_s \cdot C$ complex under basal conditions. This is what we observed. Our data support the conclusion of Tolkovsky *et al.* (1982), who proposed that in turkey erythrocyte plasma membrane G_s and C are tightly coupled. Recently Arad *et al.* (1984) have also reported that the native enzyme $G_s \cdot C$ complex has the same M_r as the p[NH]ppG-preactivated adenylate cyclase and this further supports the idea that G_s and C are tightly associated.

Purified preactivated G_s has been reported to cause a large increase in adenylate cyclase activity in human platelet membranes after a lag period (Jacob *et al.*, 1983). This may suggest that G_s is not tightly associated with the catalytic unit and can be exchanged with exogenous G_s . However, the increase in activity upon adding exogenous G_s may result from the fact that there is an excess of the catalytic unit over G_s in the membrane (see Smigel *et al.*, 1984). A further difficulty with this type of study is that the presence of non-ionic detergent (Lubrol), which is required to stabilize purified G_s , can perturb the lipid bilayer structure of the membrane (reviewed by Ross & Gilman, 1980; Houslay & Gordon, 1983) and affect the binding between G_s and the catalytic unit under basal conditions. Dissociation of G_s from catalytic unit in the presence of Lubrol has been reported (Westcott *et al.*, 1979). More recently, Smigel *et al.* (1984) have also reported a dissociation of free G_s from a stably activated adenylate cyclase in the presence of cholate. Our data suggest that, under basal conditions, G_s is tightly bound to the catalytic unit and this may account for the lag period required for the exogenous G_s to exhibit its maximal activatory effect, as obtained in Jacob *et al.* (1983). It is also possible that the G_s is more readily exchanged out from C in human platelet membrane than in the adenylate cyclase system of the rat liver membranes.

The data presented here also suggest that the conversion of inactive G_s to active G_s^* is the rate-limiting step in the activation of adenylate cyclase by GTP γ S, rather than the binding of guanine nucleotide to G_s or the interaction between G_s and C. If either the binding of GTP γ S to G_s or the interaction between G_s^* and C is rate-limiting, a decrease in the number of G_s should increase the lag time of GTP γ S activation. However, neither the photoinactivation by A-GTP nor competition with GDP β S had any effect on the lag time. Also, the increase in lag time, while K_a for GTP γ S remains

unchanged, upon lowering the temperature is another indication that the rate of activation from G_s to G_s^* is independent of the binding of GTP γ S. The rate-limiting step is the G_s to G_s^* conversion and this process is temperature sensitive.

Iyengar *et al.* (1981) examined the restoration of guanine nucleotide responsiveness to cyc $^{-}$ S49 lymphoma cell membranes by detergent extracts of liver cell plasma membranes. They found that the rate of activation by p[NH]ppG was independent of the amount of extract added, while the extent of activation was not. This further supports the view that the rate-limiting step is not the interaction between G_s and C but rather the conversion of inactive G_s to active G_s^* .

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