Activation of adenylate cyclase in human platelet membranes by guanosine 5'-[$\beta\gamma$ -imido]triphosphate is inhibited by cyclic-AMP-dependent phosphorylation

Slow activation occurs in the absence of ATP

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1. Incubation of platelet membranes with guanosine 5'-[$\beta\gamma$ -imido]triphosphate causes a slow increase in G_s (stimulatory GTP-binding protein) activation of adenylate cyclase. Mg^{2+} is necessary for this slow activation. 2. This process is inhibited in the presence of ATP, and inhibition is greater if cyclic AMP is also included in the incubation. Adenosine 5'-[$\beta\gamma$ -imido]triphosphate instead of ATP in the incubation facilitates the slow activation in the presence of cyclic AMP, and incubation of membranes with cyclic-AMP-dependent protein kinase inhibitor decreased inhibition of the slow activation of adenylate cyclase by ATP and cyclic AMP. 3. A protein of 45 kDa in platelet membranes is phosphorylated in a cyclic-AMP-dependent manner. 4. The transition from a reversibly activated form of G_s to an irreversibly activated form is substantially slower in the presence of ATP and cyclic AMP. 5. We propose that guanosine 5'-[$\beta\gamma$ -imido]triphosphate-activated G_s may exist in phosphorylated or non-phosphorylated forms, and that the non-phosphorylated form is the more active of the two species. The non-phosphorylated form of G_s may correspond to the irreversibly activated state.

INTRODUCTION

It is clear that at least three membrane components are necessary for modulation of adenylate cyclase activity by hormones. A receptor provides a site for interaction with the hormone, and either an activatory guanine nucleotide-binding protein (G_s) or an inhibitory guanine nucleotide-binding-protein (G_i) is necessary for coupling between the receptor and the catalytic unit of adenylate cyclase.

Control over the activation of cells by hormones can be exerted at several levels, and it is now apparent that phosphorylation of membrane protein components is a general mechanism by which transmembrane signalling can be modified. β -Adrenergic receptors, for example, are phosphorylated during desensitization (Sibley & Lefkowitz, 1985) and the function of the insulin receptor is also controlled by phosphorylation (Pang *et al.*, 1985; Horn *et al.*, 1986). Inactivation of G_i has been demonstrated after phosphorylation by protein kinase C (Katada *et al.*, 1985). In view of the structural and functional parallels between G_i and G_s , it might be expected that G_s function can similarly be controlled by phosphorylation. Previous work (Simpson & Pfeuffer, 1980; Stadel *et al.*, 1981) has shown that analogues of cyclic AMP induce heterologous desensitization and that G_s function is impaired, which implies that phosphorylation of G_s is involved in the inhibition of its function. Rodbell (1985) has suggested that covalent modification, including phosphorylation, of G-proteins may provide a general means of modulating their activity. To date, however, we are not aware of a direct demonstration of this mechanism in operation.

Adenylate cyclase in platelet membranes is activated by prostaglandin E_1 and inhibited by adrenaline, and provides a system in which both G_s and G_i function can be examined (Steer & Wood, 1979; Jakobs *et al.*, 1983).

G-proteins can be activated in the absence of hormonal stimulation of GTP or non-hydrolysable analogues such as guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG). The activation of adenylate cyclase by p[NH]ppG depends on its concentration and on that of Mg^{2+} (Steer & Wood, 1979; Iyengar & Birnbaumer, 1981; Northup *et al.*, 1982; Jakobs *et al.*, 1983).

In the present work we show that activation of adenylate cyclase in human platelet membranes by 10 μ M-p[NH]ppG and 2 mM- Mg^{2+} is biphasic, with a gradual rise in activity after an initial rapid activation. The slow activation of G_s can be inhibited by phosphorylation.

EXPERIMENTAL

Materials

p[NH]ppG, p[NH]ppA, GDP[S], cyclic AMP, phosphocreatine and creatine kinase were from Boehringer, Lewes, East Sussex, U.K. ATP (cat. no. A3377), protein kinase inhibitor (type II) and other materials were from Sigma. Cyclic [3 H]AMP and [32 P]P_i were from Amersham International, and [α - 32 P]ATP was synthesized by the method of Martin & Voorheis (1977). [γ - 32 P]ATP was synthesized by the method of Johnson & Walseth (1979).

Abbreviations used: p[NH]ppA, adenosine 5'-[$\beta\gamma$ -imido]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; G_i , inhibitory GTP-binding protein of adenylate cyclase; G_s , stimulatory GTP-binding protein of adenylate cyclase; G_{rev} , reversibly activated form of G_s ; G_{irr} , quasi-irreversibly activated form of G_s .

Membrane preparation

Human platelet membranes, prepared as described previously (Stein & Martin, 1983) from four packs of outdated human platelet concentrate per preparation, were stored under liquid N_2 and thawed immediately before use. Membrane protein was measured by the method of Lowry *et al.* (1951).

Membranes were diluted to about 1 mg of protein/ml and warmed to 30 °C for 8 min; the same temperature was maintained for all subsequent manipulations, and all media contained 25 mM-Tris/HCl, pH 7.4, and 1 mM-DTT.

Preincubations

These were performed as follows: typically, 25 μ l samples of membranes containing 20–50 μ g of protein were added to an equal volume of medium to give a final concentration of 10 μ M-p[NH]ppG and 2 mM- Mg^{2+} , unless otherwise specified. These were preincubated for periods of up to 20 min. Other conditions or additions are specified in the legends.

Adenylate cyclase assays

These were conducted as described previously (Wong & Martin, 1986) by adding 50 μ l of medium to the preincubations to give (final concns.) 100 μ M-ATP, 1.0×10^6 c.p.m. of [α - 32 P]ATP, 500 μ M-cyclic AMP, 0.6 mg of creatine kinase/ml and 15 mM-phosphocreatine. p[NH]ppG and Mg^{2+} were added, where appropriate, to maintain the same concentration as in the preincubation. After 20 min incubation, activity was stopped by adding 100 μ l of solution containing 2% (w/v) SDS, 40 mM-ATP and 1.4 mM-cyclic AMP. Cyclic [32 P]AMP was then determined by the method of Saloman *et al.* (1974).

Phosphorylation of platelet membranes

This was performed at 30 °C in 25 mM-Tris/HCl, pH 7.4, containing 2 mM- Mg^{2+} , 10 μ M-p[NH]ppG, 20 μ M-ATP and 5×10^6 c.p.m. of [γ - 32 P]ATP. The reaction was terminated by addition of 4 vol. of 1.25 $\times$ sample-solubilizing buffer [2% SDS, 5% mercaptoethanol, 10% glycerol (all w/v) in 6.25 mM-Tris/HCl, pH 6.8], and up to 20 μ g of protein per track was analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). Autoradiographs were prepared as described previously (Wong & Martin, 1985), and were quantified with an RFT Transidyne 2955 scanning densitometer interfaced to an Apple IIe microcomputer. Alternatively, the phosphorylated bands were cut from the gel, dissolved in 0.5 ml of 100-vol. H_2O_2 at 60 °C, and their radioactivity was measured by scintillation counting, with 7 μ l of acetic acid added to decrease luminescence of the sample.

RESULTS AND DISCUSSION

Fig. 1 shows the time course of adenylate cyclase activity in platelet membranes under basal conditions and in the presence of p[NH]ppG (10 μ M) and Mg^{2+} (2 mM). Basal cyclic AMP accumulation was linear with time, and p[NH]ppG activated with a lag of about 4 min; activation was about 3-fold.

Fig. 2(a) shows time courses for p[NH]ppG activation after preincubation with p[NH]ppG and Mg^{2+} at 30 °C for up to 10 min. Without preincubation, a similar lag

Fig. 1. Time course of basal and p[NH]ppG-activated adenylate cyclase in human platelet membranes

Tubes were set up containing 2.25 ml of the adenylate cyclase assay mixture at 30 °C as described in the Experimental section, either with (○) or without (●) 10 μ M-p[NH]ppG. The assays were initiated by pipetting into each tube 0.75 ml of pre-warmed platelet membranes. Duplicate 100 μ l samples containing 18 μ g of membrane protein were withdrawn at the indicated times thereafter (separated by 6 s) and pipetted into 100 μ l portions of the SDS/ATP/cyclic AMP stopping solution. Results shown are the means of duplicates (pmol of cyclic AMP/mg of membrane protein).

time was observed, but after either 5 or 10 min preincubation with p[NH]ppG and Mg^{2+} the lag time was abolished. Cyclic AMP accumulation was linear with time, and adenylate cyclase activity increased with preincubation time, so that after 10 min activity was about 50% higher than without preincubation. We have demonstrated that the rate-limiting step of the activation of G_s in rat liver membranes is the conversion of inactive G_s into its active form after guanine nucleotide binding (Wong & Martin, 1986). This experiment demonstrates that 5 min preincubation is sufficient for completion of this process, since the lag time is abolished after this time, but that a further, slow, p[NH]ppG-dependent activation occurs during longer preincubation. Fig. 2(b) shows the increase in adenylate cyclase activity assayed for 20 min after up to 20 min preincubation with p[NH]ppG. The slow phase of activation resulted in an increase of about 100% over the activation during the rapid phase.

The activation of G_s is Mg^{2+} -dependent (Iyengar & Birnbaumer, 1981). The Mg^{2+} -dependence of the slow activation is shown in Fig. 3, which demonstrates an absolute requirement for Mg^{2+} . The optimal Mg^{2+} concentration is about 1 mM.

The observed adenylate cyclase activity in a plasma-membrane preparation is thought to reflect the balance between the activatory effects of G_s and the inhibitory effects of G_i (Cerione *et al.*, 1985; Northup, 1985). We considered the possibility that the observed rise in adenylate cyclase activity in platelet membranes might be caused by a relative decline in G_i activity during

Fig. 2. Time course of p[NH]ppG-activated adenylate cyclase after preincubation in the presence of p[NH]ppG

(a) Three tubes were set up containing 0.75 ml of p[NH]ppG. To the first (○), 1.5 ml of assay mixture was added, and all were warmed to 30 °C. Incubations were initiated by adding 0.75 ml of pre-warmed platelet membranes to each tube. After either 5 (■) or 10 (△) min preincubation, 1.5 ml of assay mixture was added to the second and third tubes. Each tube was sampled by transferring 100 μ l samples (containing 51 μ g of membrane protein) at the indicated times to an equal volume of the SDS/ATP/cyclic AMP stopping solution. Single determinations are plotted of cyclase activation, expressed in pmol of cyclic AMP/mg of membrane protein. (b) Platelet membranes (48 μ g of protein/tube) in 50 μ l were preincubated for the indicated times with 10 μ M-p[NH]ppG and 2 mM-Mg²⁺, and then the complete assay mixture was added in an equal volume of medium. Assays were for 20 min, as described in the Experimental section.

incubation with p[NH]ppG. However, Fig. 4 shows that the inhibitory response was maintained during incubation with p[NH]ppG: addition of adrenaline (10 μ M) resulted in lower adenylate cyclase activities (about 10 pmol of AMP/min per mg of protein) throughout the time course. This suggests that G_i function was not impaired during the preincubation, and that the net rise in adenylate cyclase activity was not caused by a decline in G_i activity.

Fig. 3. Mg²⁺-dependence of the slow activation of platelet membrane adenylate cyclase by p[NH]ppG

Platelet membranes (36 μ g/tube) were preincubated with 10 μ M-p[NH]ppG and the indicated concentration of Mg²⁺ for 20 min. Triplicate adenylate cyclase determinations were then performed as described in the Experimental section at 50 mM-Mg²⁺. Initial cyclase activity without preincubation was also determined. Results are expressed as a percentage of the maximum rise in activity observed after preincubation. Values are means $\pm$ S.E.M. for three separate experiments.

The linearity of the assay time course in Figs. 1 and 2 shows that further activation of cyclase activity does not occur in the presence of the complete assay mixture. In addition to the preincubation components, the assay contained ATP, an ATP-regenerating system and

Fig. 4. Time course of p[NH]ppG-activation of platelet adenylate cyclase: assay of inhibitory response to adrenaline

Preincubations were set up at 30 °C, containing 10 μ M-p[NH]ppG. At the indicated times, 100 μ l samples (containing 52 μ g of membrane protein) were withdrawn for adenylate cyclase assay. For these experiments, the assay tubes were kept on ice before use to prevent degradation of adrenaline, and assay was commenced by dispensing warmed portions of membrane suspension into these, and then incubating at 30 °C for 20 min as described in the Experimental section. Activities shown (pmol of cyclic AMP/min per mg of membrane protein), without (○) or with (▲) 10 μ M-adrenaline, are means $\pm$ S.E.M. for triplicate determinations.

Fig. 5. Inhibition of p[NH]ppG activation of platelet adenylate cyclase by assay components

Two tubes were set up at 30 °C containing p[NH]ppG, either without (○) or with (□) 100 μM-ATP and 500 μM cyclic AMP (final concn.). Preincubations were initiated by pipetting in pre-warmed membranes. At the indicated times, triplicate samples (containing 71 μg of membrane protein) were withdrawn at 6 s intervals for adenylate cyclase assay as described in the Experimental section. The value shown are means ± S.E.M. for triplicate assays, expressed in pmol of cyclic AMP/min per mg of membrane protein.

cyclic AMP. Fig. 5 shows that addition of these components to the preincubation substantially inhibited the slow rise in adenylate cyclase activity.

Table 1 shows that inclusion of p[NH]ppA, a non-hydrolysable analogue of ATP, in the preincubation potentiated activation by p[NH]ppG, whereas ATP inhibited as in Fig. 5. This suggests that a transferable terminal phosphate group is essential for the inhibition of the slow p[NH]ppG-dependent rise in adenylate cyclase activity, and also suggests that some endogenous phosphorylation may have occurred in the absence of added ATP.

Katada *et al.* (1985) have shown that Ca²⁺-dependent

phosphorylation of G_i (by protein kinase C) inhibited its activity. In our experiments, however, the assays were conducted in the absence of Ca²⁺; the addition of 0.4 mM-EGTA did not modify adenylate cyclase activity (results not shown). The presence of cyclic AMP during the assay suggests that the ATP-dependent inhibition of the slow activation might result from the action of a cyclic-AMP-dependent protein kinase, and the degree of inhibition observed at 10 μM or 50 μM-cyclic AMP was similar to that seen with 500 μM-cyclic AMP as in Fig. 5 (results not shown). Table 2 shows that cyclic AMP increased the ATP-dependent inhibition of the slow p[NH]ppG-dependent rise in cyclase activity, but had no effect on the slow activation in the absence of ATP. Some inhibition was observed when ATP was added in the absence of cyclic AMP. Adenylate cyclase activity under these conditions is sufficient to generate micromolar concentrations of cyclic AMP after a few minutes, so that it is possible that the partial inhibition observed with ATP alone is also cyclic-AMP-dependent.

Pretreatment of platelet membranes with 2.5 mM-Mg²⁺, 100 μM-cyclic AMP and 100 μg of the cyclic AMP-dependent protein kinase inhibitor/ml decreased inhibition of the slow activation in the presence of ATP and cyclic AMP (Table 3).

We considered the possibility that GTP contaminating the ATP used might be responsible for inhibition of the slow activation of adenylate cyclase; it is established (Wong & Martin, 1985) that GTP slows the activation of G_s by p[NH]ppG in liver membranes. However, the addition of up to 1 μM-GTP during preincubation of platelet membranes did not impair the slow activation of adenylate cyclase (results not shown). Also, lower concentrations of ATP supported the same degree of inhibition of the slow activation as 100 μM-ATP, and a purer grade of ATP (Sigma cat. no. A2383) gave results indistinguishable from the rest of the present work. This evidence supports our view that GTP or other contaminants were not responsible for the effects of ATP on the slow activation described above.

From the above experiments, it seems likely that cyclic AMP-dependent phosphorylation of a membrane component inhibits the slow p[NH]ppG-dependent activation of adenylate cyclase, and that a likely phosphate

Table 1. Effect of p[NH]ppA in preincubation

Platelet membranes (32 μg of protein per tube) were preincubated for the specified times in the presence of 10 μM-p[NH]ppG, 2 mM-Mg²⁺ and 50 μM-cyclic AMP either with no other additions (Control) or with 100 μM-p[NH]ppA. Adenylate cyclase activity (pmol of cyclic AMP/min per mg of membrane protein) was then assayed for 20 min as described in the Experimental section, but at a final concentration of 500 μM-ATP and 50 μM-p[NH]ppA for each group. Values shown are means ± S.E.M. for triplicate determinations.

Preincubation time (min) . . .	Adenylate cyclase activity			
	0	5	10	20
Control	192 ± 4	236 ± 2	238 ± 10	255 ± 13
p[NH]ppA		225 ± 11	256 ± 13	291 ± 15

Table 2. Effect of cyclic AMP and ATP on cyclase activation of p[NH]ppG

Platelet membranes were preincubated for the specified times in the presence of 10 μM-p[NH]ppG, 1 mM-Mg²⁺, 1 mM-theophylline and no other additions (Control) or with 50 μM-cyclic AMP and 100 μM-ATP either alone or in combination. Adenylate cyclase activity (20 μg of protein per tube) was then determined for 20 min as described in the Experimental section. Results (means ± S.E.M. for triplicate determinations) are expressed in pmol of cyclic AMP/min per mg of membrane protein.

Preincubation time (min) . . .	Adenylate cyclase activity			
	0	5	10	20
Control	43 ± 1	95 ± 2	104 ± 4	99 ± 1
Cyclic AMP		95 ± 1	103 ± 1	94 ± 1
ATP		69 ± 2	85 ± 1	93 ± 1
ATP + cyclic AMP		69 ± 2	79 ± 1	82 ± 2

Table 3. Effect of protein kinase inhibitor on p[NH]ppG activation of adenylate cyclase in the presence of ATP and cyclic AMP

Platelet membranes (500 μg) were incubated at 30 °C for 15 min in medium containing 2.5 mM- Mg^{2+} , 100 μM -cyclic AMP, without (Control I) or with (PKI) 100 μg of the inhibitor of cyclic-AMP-dependent protein kinase/ml. The membranes were then diluted to 1 ml with cold Tris buffer, centrifuged (15000 g -min) and resuspended to 350 μl as described in the Experimental section: 12 μg of protein was determined in triplicate before and after preincubation with 10 μM -p[NH]ppG and 1 mM- Mg^{2+} , either with or without 50 μM -cyclic AMP and 100 μM -ATP present during the preincubation as indicated above. For Control II, membranes were held on ice, and then their cyclase activity was similarly determined. Results are expressed as the ratio of cyclase activity after 20 min preincubation to the initial activity: *significantly lower cyclase activity in the presence of ATP and cyclic AMP than in their absence ($P < 0.05$).

	Relative cyclase activity- after pre-incubation with or without ATP and cyclic AMP	
	Without	With
Control I	1.77	1.43*
Control II	1.88	1.46*
PKI	1.75	1.72

acceptor might be G_s itself. Fig. 6 shows the incorporation of phosphate from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into platelet membrane proteins in the presence and absence of cyclic AMP. Four major bands exhibited cyclic AMP-dependent phosphorylation. One of these was of approx. 45 kDa, consistent with the α -subunit of G_s . The incorporation of ^{32}P into this band was increased 2-fold by including cyclic AMP during phosphorylation, as estimated by densitometry of the autoradiographs. The phosphorylation of the 45 kDa band was inhibited by cyclic AMP-dependent protein kinase inhibitor (10 μg /tube, sufficient to inhibit the transfer of 6 pmol of phosphate/min), thus confirming that its phosphorylation was cyclic AMP-dependent (results not shown). Phosphorylation of several other bands was also inhibited, although some appeared to be phosphorylated by other protein kinases. Substantial phosphorylation of the 45 kDa band occurred at zero time; this is consistent with our finding (Fig. 5) that ATP- and cyclic-AMP-dependent inhibition of the slow activation occurred without an apparent lag during preincubation of platelet membranes with p[NH]ppG. The dependence of phosphorylation on preincubation time remains to be determined.

The extent of incorporation of phosphate into the 45 kDa band under the present conditions was about 3 pmol/mg of membrane protein. This is similar to previous estimates of G_s contents in plasma membranes from pigeon erythrocytes, as measured by ADP-ribosylation by cholera toxin (8 pmol/mg; Cassel & Pfeuffer, 1978), or from purification data on human erythrocytes (4 pmol/mg; Codina *et al.*, 1984a) or rabbit liver (16 pmol/mg; Sternweis *et al.*, 1981). We are not aware of a previous estimate for the G_s content of platelet membranes.

Fig. 6. Cyclic-AMP-dependence of phosphorylation of platelet membrane proteins

Tubes were set up containing 10 μM -p[NH]ppG, 2 mM- Mg^{2+} and 5×10^6 c.p.m. of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, either alone (a and b) or with 20 μM -ATP added (c and d); 500 μM -cyclic AMP was absent (a and c) or present (b and d). Phosphorylation was initiated by adding 123 μg of membrane protein to a final volume of 100 μl . After 20 min the reaction was stopped by adding 400 μl of sample-solubilizing buffer, and SDS/polyacrylamide-gel electrophoresis was used to separate membrane proteins as described in the Experimental section: 12 μg of protein was loaded on to each track, and autoradiography was performed as described in the Experimental section. Positions of M_r markers, specified in the centre, are indicated by arrows to left and right of the Figure.

The activation of G_s by p[NH]ppG in rat liver plasma membranes is biphasic; a transition occurs from a reversibly activated form, G_{rev} , to an irreversibly activated form, G_{irr} , on prolonged incubation with p[NH]ppG. The two forms of G_s are equally effective in activating the catalytic subunit of adenylate cyclase (Wong & Martin, 1986). We examined the development of an irreversibly activated form of G_s in platelet membranes by including a 10-fold excess of GDP[S] during the adenylate cyclase assay after different times of preincubation with 10 μM -p[NH]ppG. GDP[S] inhibited p[NH]ppG activation of adenylate cyclase by 90% when the two guanine nucleotides were added to the adenylate cyclase assay at the same time. However, GDP[S] had virtually no effect on the activity of adenylate cyclase in membranes that had been preincubated for 20 min in the absence of ATP and cyclic AMP (Table 4). This raises the possibility that, in platelets, G_{rev} and G_{irr} are not equally effective in activating adenylate cyclase, and that the slow

Table 4. Reversibility of p[NH]ppG activation of cyclase by GDP[S]

Platelet membranes (32 μ g of protein per tube) were preincubated for the specified times in the presence of 10 μ M-p[NH]ppG and 2 mM-Mg²⁺. Adenylate cyclase activity (pmol of cyclic AMP/min per mg of membrane protein) was then determined for 20 min as described in the Experimental section, but either with or without (Control) 100 μ M-GDP[S] present during the assay. Values shown are means $\pm$ S.E.M. for triplicate determinations

Preincubation time (min) . . .	Adenylate cyclase activity		
	0	5	20
Control	63.6 $\pm$ 2.4	85.6 $\pm$ 6.8	92.7 $\pm$ 2.9
GDP[S]	4.9 $\pm$ 0.7	77.6 $\pm$ 2.4	90.8 $\pm$ 4.6

Fig. 7. Effect of ATP with cyclic AMP on the time course of development of G_{irr}

Experiments similar to that described for Table 4 were performed to estimate the irreversibility of G_s activation after the indicated times of preincubation with p[NH]ppG. The percentage adenylate cyclase activity assayed with GDP[S] relative to activity without GDP[S] is plotted either without (○) or with (□) 100 μ M-ATP and 50 μ M-cyclic AMP present during the preincubation. The vertical broken lines indicate $t_{1/2}$ (calculated assuming that 100% irreversibility will eventually be reached for both curves) for the development of G_{irr} . Points are the mean values from three experiments.

activation corresponds to the transition between the two states of G_s .

Fig. 7 shows the effects of ATP together with cyclic AMP on the onset of irreversibility during preincubation with p[NH]ppG. Preincubation with p[NH]ppG rapidly abolished the ability of GDP[S] to reverse adenylate cyclase activation. If ATP and cyclic AMP were included in the preincubation, however, substantial reversibility was maintained throughout the time course examined, the half-time for the transition increasing from 90 s to more than 5 min.

General discussion

In the present work we have shown that basal adenylate cyclase activity in platelet membranes is constant; the accumulation of cyclic AMP with time is linear (Fig. 1). When activated by p[NH]ppG in the assay mixture with ATP and cyclic AMP, adenylate cyclase activity increases with a lag time of about 5 min, and thereafter constant activity persists. In contrast, preincubation with p[NH]ppG and Mg²⁺ in the absence of ATP and cyclic AMP results in a further increase in adenylate cyclase activity over at least 20 min. The rise in adenylate cyclase activity is not the result of loss of G_i activity during the preincubation at 30 °C, since inhibition by adrenaline is unimpaired (Fig. 4). The effect can be ascribed to slow activation of G_s . Steer & Wood (1979), also using p[NH]ppG and human platelet membranes, and Northup *et al.* (1982) and Codina *et al.* (1984b), using guanosine 5'-[γ -thio]triphosphate and p[NH]ppG with purified G_s , similarly demonstrated slow activation of G_s .

Jakobs *et al.* (1983) found it necessary to equilibrate platelet membranes with otherwise complete assay medium to establish linear conditions in adenylate cyclase assays before adding [α -³²P]ATP. In the present work, ATP and cyclic AMP included in preincubations inhibited the slow activation of adenylate cyclase (Table 2 and Fig. 5).

These observations suggest that cyclic-AMP-dependent phosphorylation of some membrane component can inhibit the slow activation of G_s , which is supported by the ability of p[NH]ppA to facilitate slow activation in the presence of cyclic AMP (Table 1). This suggests that a feedback-inhibitory mechanism at the level of G_s may control activation of adenylate cyclase. A similar inhibitory pathway has been demonstrated in platelet membranes (Katada *et al.*, 1985), which is not cyclic-AMP-dependent; protein kinase C phosphorylates the α -subunit of G_i and blocks its inhibitory action on adenylate cyclase; G_s was not a substrate for phosphorylation by protein kinase C. Jakobs *et al.* (1985) showed that this mechanism also modulates the response to inhibitory hormones in intact platelets.

We show that platelet membrane components are phosphorylated in a cyclic AMP-dependent fashion (Fig. 6). One of the major substrates for phosphorylation under these conditions is of 45 kDa. These findings suggest that the 45 kDa substrate for phosphorylation might be the α -subunit of G_s . Its identity remains to be confirmed, however. Enyedi *et al.* (1983) have demonstrated cyclic AMP-dependent phosphorylation of a protein of this size in both platelet and lymphocyte membranes, suggesting that it is not unique to platelets, although Marchmont & Houslay (1980) did not report phosphorylation of a 45 kDa protein in rat liver membrane preparations under similar conditions.

Phosphorylation of membrane components is now recognized as a means of regulating their activity, and has been implicated in desensitization of hormone-sensitive adenylate cyclase (Sibley & Lefkowitz, 1985). The coupling of β -adrenergic receptors to adenylate cyclase is impaired by cyclic-AMP-dependent phosphorylation (Sibley *et al.*, 1984; Benovic *et al.*, 1985), and other observations of decreased sensitivity to hormones have been made without loss of receptor-binding activity (Simpson & Pfeuffer, 1980; Stadel *et al.*, 1981). Their

work showed that incubating cells with cyclic AMP analogues caused similar heterologous desensitization, suggesting that G-proteins are also phosphorylated in a cyclic-AMP-dependent manner, leading to a partial loss of adenylate cyclase activity. Rich *et al.* (1984), using MDCK cells, concluded that the basis for such heterologous desensitization might be greater availability of G_i . An increased negative charge, consistent with a single phosphorylation, was demonstrated by Schleifer *et al.* (1980), on G_s from the UNC mutant of the S49 murine lymphoma cell, suggesting that the lack of G_s activity in these cells might be caused by this covalent modification of G_s . Our observations provide support for this view; our results are consistent with phosphorylation of the active α -subunit of G_s causing partial inactivation. This would suggest that the functional lesion of UNC S49 cells might include a lack of the phosphatase activity necessary to activate the free α -subunit.

Activation of G_s by p[NH]ppG can be partially reversed by the addition of GDP[S] during the adenylate cyclase assay (Table 3). The onset of irreversibility of G_s activation is sufficiently slow to suggest that it may be a manifestation of the slow activation of G_s described above. Inclusion of ATP and cyclic AMP substantially slows both processes (Figs. 5 and 7), suggesting that the two events might be subject to the same mechanism. This would imply that the effectiveness of G_{irr} in activating adenylate cyclase is considerable greater than that of G_{rev} , which is not the case in rat liver membranes (Wong & Martin, 1986). If the phosphorylated 45 kDa protein is indeed G_s (which requires further study), our work provides evidence for the following scheme for activation of G_s :

It is clear that unequivocal identification of the 45 kDa band is necessary, and that further examination of the dephosphorylation of G_s may permit kinetic comparison with the slow activation of adenylate cyclase described in the present work. The cellular locus of the kinase and phosphatase activity (and control of the latter) responsible for interconversion of the two states of G_s also needs to be determined. A similar two-stage activation process of G_s was suggested by Codina *et al.* (1984b), where the equilibrium between states of G_s , having different sedimentation coefficients, was modified by the incubation temperature. The relationship between the present work and those observations remains to be elucidated.

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