Evidence for regulation of human platelet adenylate cyclase by phosphorylation

Inhibition by ATP and guanosine 5'-[β -thio]diphosphate occur by distinct mechanisms

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1. Incubation of human platelet membranes with guanosine 5'-[β -imido]triphosphate (p[NH]ppG) causes a time-dependent increase in the activation of adenylate cyclase due to G_s (the stimulatory GTP-binding protein). Forskolin enhances adenylate cyclase activity but does not interfere with the process of activation. The activation follows first-order kinetics in both the presence and the absence of the assay components. 2. ATP in the presence or the absence of an ATP-regenerating system of phosphocreatine and creatine kinase inhibits activation. 3. Hydrolysis of ATP to ADP does not lead to receptor-mediated inhibition of adenylate cyclase acting via G_i (the inhibitory GTP-binding protein). The ADP analogue adenosine 5'-[β -thio]diphosphate (ADP[S]) does not inhibit the activation process. 4. Phosphocreatine alone inhibits adenylate cyclase activation at concentrations above 1 mM. 5. Inhibition by phosphocreatine is not due to the chelation of free Mg^{2+} ions. 6. Inhibition by ATP and the other assay components occurs throughout the activation process, decreasing both the rate of activation and the maximum activity obtained. 7. Maximal activation of adenylate cyclase after prolonged incubation with p[NH]ppG slowly reverses in the presence of the assay components. 8. A 10-fold excess of the GDP analogue guanosine 5'-[β -thio]diphosphate (GDP[S]) over p[NH]ppG inhibits the activation process completely, at all stages of the time course. 9. Preincubations in the presence and absence of ATP, cyclic AMP, phosphocreatine and creatine kinase show equal sensitivity to increasing GDP[S] concentration. These data show that the inhibition observed in the presence of ATP is not due to endogenous or contaminating guanine nucleotides, and suggest that phosphoryl transfer may regulate adenylate cyclase activity.

INTRODUCTION

A variety of agonists act on adenylate cyclase to modulate levels of the intracellular second messenger cyclic AMP. This is achieved by the coupling of activated, ligand-occupied receptors to either stimulatory (G_s) or inhibitory (G_i) guanine-nucleotide-binding proteins (G-proteins). In turn, these proteins respectively increase or decrease the activity of the catalytic unit of adenylate cyclase and thus regulate the production of cyclic AMP.

G-proteins are heterotrimers of α , β and γ subunits. Activation is thought to be initiated by the exchange on the α subunit of bound GDP for free GTP. This occurs as the result of interaction of the G-protein with an activated ligand-receptor complex. Subsequent to GTP binding, the G-protein is thought to dissociate into α and $\beta\gamma$ moieties, and it is the α subunit of G_s which in turn stimulates the catalytic unit. The activation is terminated by the hydrolysis of GTP by the α subunit, GDP remaining bound to $G_s\alpha$ and inorganic phosphate being released. This is followed by the re-association of the α and $\beta\gamma$ subunits (for a review, see Gilman, 1987).

Activation of adenylate cyclase can be achieved by the use of analogues of GTP such as guanosine 5'-[β -imido]triphosphate (p[NH]ppG) and guanosine 5'-[γ -thio]triphosphate (GTP[S]), which cause persistent activation of G-proteins, since the terminal phosphate group is resistant to hydrolysis (Harwood *et al.*, 1973; Londos *et al.*, 1974). In most systems, activation by GTP analogues occurs in the absence of receptor stimulation and this has been exploited in order to study how activated G-proteins interact with the catalytic unit of adenylate cyclase. The study of inhibition of G-proteins by GDP is complicated by its tendency

to be phosphorylated to GTP in the adenylate cyclase assay system. However, this does not occur with the GDP analogue guanosine 5'-[β -thio]diphosphate (GDP[S]). GDP[S] acts by occupying the guanine-nucleotide-binding site on G-proteins, inhibiting or reversing activation by competing with GTP and its analogues such as p[NH]ppG (Eckstein *et al.*, 1979).

Early experiments to assay adenylate cyclase activity in membrane preparations used millimolar concentrations of ATP (Rodbell, 1967; Krishna *et al.*, 1968), as constant activity of the catalytic unit of adenylate cyclase depends on the maintenance of the ATP concentration during the assay period. However, ATP is quickly degraded by the action of membrane ATPases, and so an ATP-regenerating system is routinely added, usually phosphocreatine and creatine kinase (Birnbaumer *et al.*, 1969). Lower concentrations of ATP, for example 100 μ M-ATP as used in most of this study, necessitate the use of a regenerating system, as the K_m of adenylate cyclase for ATP is of the order of 50 μ M (Lin *et al.*, 1975).

Activation of adenylate cyclase by p[NH]ppG is dependent on the concentrations of both p[NH]ppG and Mg^{2+} (Steer & Wood, 1979; Iyengar & Birnbaumer, 1981; Northup *et al.*, 1982; Jakobs *et al.*, 1983). The activation process has previously been characterized as consisting of an initial lag period, the duration of which varies from about 30 s to several minutes depending on the membrane preparation, followed by sustained constant adenylate cyclase activity. This pattern of activation of adenylate cyclase has been observed in many different membrane preparations (Rodbell *et al.*, 1975), including human platelet membranes (Jakobs *et al.*, 1983; Farndale *et al.*, 1987).

Previous work in this laboratory found that human platelet

Abbreviations used: ADP[S], adenosine 5'-[β -thio]diphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; G_i , inhibitory GTP-binding protein of adenylate cyclase; G_s , stimulatory GTP-binding protein of adenylate cyclase; DTT, dithiothreitol.

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adenylate cyclase responds differently to p[NH]ppG (Farndale *et al.*, 1987). Pre-exposure of platelet plasma membranes to p[NH]ppG (10 μ M) at approximately physiological levels of Mg^{2+} (1 mM) led to a doubling of adenylate cyclase activity, which was subsequently determined by transferring membranes from the preincubation to an assay containing ATP, cyclic AMP, phosphocreatine and creatine kinase. The development of this activity took about 20 min and was called the 'slow activation'. The activation was markedly decreased when ATP, cyclic AMP, phosphocreatine and creatine kinase were also included in the preincubation. These are the same conditions under which adenylate cyclase assays are normally carried out, where high concentrations of cyclic AMP are included to prevent degradation of [32 P]cyclic AMP by phosphodiesterase activity. The ability of p[NH]ppG to activate human platelet adenylate cyclase appears to be closely linked to the environment in which the membranes are exposed to p[NH]ppG. This effect was shown to be due to increased activity to G_s , and not to a decrease in G_i activity (Farndale *et al.*, 1987).

Recently it has become evident that covalent modification by phosphorylation provides a means of modulating the activities of the different components of the adenylate cyclase complex. This mechanism of regulation could in principle be exerted at the level of the receptor, G-proteins or catalytic unit. Receptor phosphorylation has been widely documented as being involved with both homologous and heterologous desensitization (reviewed by Sibley *et al.*, 1987). Two possible phosphorylation sites on the catalytic unit have been identified from a cDNA sequence from bovine brain, one for cyclic AMP-dependent protein kinase and the other for protein kinase C (Krupinski *et al.*, 1989). There is also some direct evidence for protein kinase C-mediated phosphorylation of the catalytic unit (Yoshimasa *et al.*, 1987). Phosphorylation of G-proteins by protein kinase C has been proposed as a likely mechanism of regulation (Katada *et al.*, 1985; Pyne *et al.*, 1989; Sagi-Eisenberg, 1989). Katada *et al.* (1985) proposed that phosphorylation of G_i caused attenuation in the inhibition of adenylate cyclase by a variety of hormones. The kinase responsible was thought to be protein kinase C, as pretreating the platelets with phorbol esters lowered the sensitivity to inhibition. More recent work using similar conditions identified the phosphorylated species as a 40 kDa pertussis-toxin-insensitive G-protein (Carlson *et al.*, 1989) which is quite distinct from the three G_i species and has been called G_z (Fong *et al.*, 1988). However, a 41 kDa phosphoprotein has been reported to react with a G_i -specific antiserum in intact platelets (Crouch & Lapetina, 1988).

There has been less interest in G_s phosphorylation. The inhibitory action of ATP and the other assay components on the activation of human platelet adenylate cyclase by p[NH]ppG described above is consistent with the idea that a change in the phosphorylation state of G_s may modulate its function. We suggested that the activation by p[NH]ppG involved phosphatase activity and the inhibition observed with ATP, cyclic AMP, phosphocreatine and creatine kinase was conversely due to phosphorylation. A possible substrate for this regulation was proposed to be G_s (Farndale *et al.*, 1987).

In the present study, the activation of human platelet adenylate cyclase by p[NH]ppG was investigated, to establish how the degree of activation obtained was dependent on the different conditions employed. In particular, ATP was found to be an inhibitor of activation. The kinetics of the activation process were measured in both the presence and the absence of ATP and a regenerating system of phosphocreatine and creatine kinase. This work provides evidence that the process of adenylate cyclase activation is reversible under those conditions previously assumed to support a constant rate of activation of adenylate cyclase. This

suggests that one or more of the steps in the sequence of adenylate cyclase activation may be due to, or regulated by, an alteration in phosphorylation state.

EXPERIMENTAL

Materials

Creatine kinase, phosphocreatine, cyclic AMP, ATP, p[NH]ppG, GDP[S] and ADP[S] were from Boehringer Mannheim, Lewes, E. Sussex, U.K., and other materials were from Sigma. Cyclic [3 H]AMP and [32 P] P_i were purchased from Amersham International. [α - 32 P]ATP was synthesized by the method of Martin & Voorheis (1977). Human platelet concentrate was a gift from the Regional Blood Transfusion Service, Hills Road, Cambridge, U.K.

Preparation of human platelet plasma membranes

Plasma membranes were prepared by a modification of the method of Stein & Martin (1983) using four packs of human platelet concentrate per preparation. The platelets were separated from contaminating erythrocytes as described previously, then resuspended in ice cold hypo-osmotic buffer (5 mM-EDTA/5 mM-Tris/HCl, pH 7.4), left on ice for 30 min and ruptured by freeze-thawing. The plasma membrane fraction was washed and purified as described previously (Stein & Martin, 1983), and aliquots were stored under liquid N_2 or at -80°C . The protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard, and varied between 7.5 and 18.5 mg/ml.

Preincubations of plasma membranes

Membranes were diluted to about 1 mg/ml into Tris buffer [25 mM- or 50 mM-Tris/HCl, pH 8.0, and 1- or 2 mM-dithiothreitol (DTT)] and warmed to 30°C for 5 min; the same temperature was maintained for all subsequent manipulations. Preincubations were started by the addition of prewarmed membranes to an equal volume of warmed preincubation mix to give final concentrations of 25 mM-Tris/HCl, pH 8.0, and 1 mM-DTT in a total volume of about 2 ml. Typically preincubations also contained 10 μ M-p[NH]ppG and 1 mM- Mg^{2+} , although other conditions are specified in the appropriate Figure legends. The resultant protein concentration varied between 0.3 and 1.0 mg/ml. After the relevant duration of preincubation, between 2 and 30 min, 50 μ l samples were removed for assay of adenylate cyclase activity.

Assay of adenylate cyclase activity in isolated plasma membranes

Adenylate cyclase activity was assayed by the method of Salomon *et al.* (1974). Assays were carried out in 25 mM-Tris/HCl, pH 8.0, this being the optimal pH for platelet adenylate cyclase activity in these membrane preparations (results not shown). The other components of the assay system were 1 mM-DTT, 1 or 10 mM- $MgCl_2$, 0.5 mM-cyclic AMP, 0.1 or 0.5 mM-ATP and 1×10^6 c.p.m. of [α - 32 P]ATP. An ATP-regenerating system of 8 mM-phosphocreatine and 0.3 mg of creatine kinase/ml was included in each assay. This was sufficient to maintain the level of ATP at more than 90% of its initial concentration over a period of 60 min at 30°C (results not shown). The total assay volume was 100 μ l, containing 15–45 μ g of membrane protein. The reaction was started on the addition of prewarmed or preincubated membranes. All assays were carried out for 5 min and then terminated by adding 100 μ l of stop solution [2% (w/v) SDS, 1.4 mM-cyclic AMP, 40 mM-ATP], and an internal standard of cyclic [3 H]AMP was added to each sample (0.1 μ Ci in a volume of 100 μ l).

Calculation of apparent stability constants

Apparent stability constants for the chelation of Mg^{2+} by phosphocreatine and ATP at pH 8.0 were calculated using a program based on that of Fabiato & Fabiato (1979) written by Dr. M. D. Brand, Department of Biochemistry, University of Cambridge, for a BBC B microcomputer. Values for stability constants were obtained from Dawson *et al.* (1986).

RESULTS AND DISCUSSION

The activation of adenylate cyclase observed after exposure of platelet membranes to p[NH]ppG and Mg^{2+} is thought to be due to a change in the activity state of G_s . As a means of testing this, the effect of forskolin was examined. Forskolin is a potent activator of adenylate cyclase in many different tissues (for a review see Daly, 1984). Activation is thought primarily to result from direct stimulation of the catalytic unit (Seamon & Daly, 1981), although stabilization of the interaction between G_s and the catalytic unit may also occur (Wong & Martin, 1983; Yamashita *et al.*, 1986). Membranes were preincubated with Mg^{2+} (1 mM) and p[NH]ppG (10 μ M) with or without forskolin (10 μ M), and the resulting adenylate cyclase activity was assayed with and without forskolin (Fig. 1).

No activation of adenylate cyclase was observed on preincubation in the presence of Mg^{2+} alone, but there was a slow decline in both basal and stimulated activity, probably due to the thermal lability of the catalytic unit of adenylate cyclase. In the presence of p[NH]ppG and Mg^{2+} , the characteristic slow increase in adenylate cyclase activity was observed, as described previously (Farndale *et al.*, 1987). The inclusion of forskolin with p[NH]ppG and Mg^{2+} in the preincubation and the subsequent assay increased the level of adenylate cyclase activity about 5-fold under all preincubation conditions, but did not alter the pattern of activation resulting from preincubation with p[NH]ppG and

Fig. 1. Forskolin enhances adenylate cyclase activity but does not interfere with the kinetics of activation of platelet adenylate cyclase by p[NH]ppG

Four preincubations were set up at 30 °C and initiated by the addition of prewarmed platelet membranes. At the indicated times, triplicate 50 μ l samples (containing 22 μ g of protein/tube) were withdrawn at 6 s intervals for adenylate cyclase assay as described in the Experimental section. The conditions for each experiment were: only 1 mM- Mg^{2+} present in both preincubation and assay (○); 1 mM- Mg^{2+} and 10 μ M-p[NH]ppG present throughout the duration of the experiment, but the adenylate cyclase activity assayed in the presence (□) or absence (●) of 10 μ M-forskolin; or 1 mM- Mg^{2+} , 10 μ M-p[NH]ppG and 10 μ M-forskolin present in both preincubation and assay (■). Activities shown are means $\pm$ S.E.M. for triplicate determinations.

Mg^{2+} . Very similar adenylate cyclase activities were recorded when forskolin was included only in the assay period but was omitted from the preincubation with p[NH]ppG and Mg^{2+} . The slow rise in adenylate cyclase activity was entirely dependent upon the presence of p[NH]ppG in the preincubation. The presence of forskolin and Mg^{2+} alone in the preincubation caused a very rapid activation of adenylate cyclase; the activity then gradually declined during the rest of the experiment, probably also due to thermal denaturation of the catalytic unit (results not shown). These data suggest that the activation of adenylate cyclase obtained on incubating human platelet membranes in the presence of p[NH]ppG is due to a change in the activity of G_s , rather than to modification of the activity of the catalytic unit.

The effect of including ATP, cyclic AMP and the ATP-regenerating system on the activation of adenylate cyclase was examined by comparing the activation kinetics under the two different sets of conditions. Fig. 2(a) shows the pooled results from four experiments in which membranes were preincubated for increasing times and the adenylate cyclase activity was then assayed. Membrane preincubations were carried out with p[NH]ppG and Mg^{2+} in the presence or absence of ATP (100 μ M), cyclic AMP (50 μ M), phosphocreatine (8 mM) and creatine kinase (0.3 mg/ml). The inclusion of these extra components in the preincubation was clearly inhibitory, slowing the activation process and also decreasing the maximum activity obtained over this time interval. The data were then further analysed to determine whether the two activation profiles had first-order kinetics (Fig. 2b). The data from the two types of preincubation produced good-fit straight lines, indicating that a first-order reaction scheme could be assigned to each. The values for the rate constants show that in the presence of ATP, cyclic AMP, phosphocreatine and creatine kinase, activation by p[NH]ppG and Mg^{2+} was severely inhibited, the half-time for the reaction increasing from 4.1 min to 7.5 min (Table 1). The rate of activation of platelet adenylate cyclase by p[NH]ppG is therefore critically dependent on the experimental environment.

The effect of ATP in the absence of the regenerating system was then examined (Fig. 3). Membranes were preincubated with Mg^{2+} (1 mM) and p[NH]ppG (10 μ M). ATP (100 μ M) was also included in certain of the preincubations with and without cyclic AMP and the phosphocreatine/creatine kinase ATP-regenerating system. The adenylate cyclase activity was assayed in the presence of the regenerating system at 10 mM- Mg^{2+} and 500 μ M-ATP. The time-dependency of the activation process was unaffected by this change in assay conditions (results not shown). The high Mg^{2+} concentration was present to increase the adenylate cyclase activity during the short assay and therefore the radioactivity recovered in each sample. ATP (500 μ M) was employed to minimize variations in the ATP concentration of the assays. Platelet membranes in the absence of an ATP-regenerating system quickly degrade ATP by the action of endogenous ATPases (results not shown). Consequently, the precise amount of ATP carried over to the assay from a preincubation without a regenerating system was unknown, so the final ATP concentration in the assay could have varied between 450 and 500 μ M. To test if this had a significant effect, membranes from control preincubations were assayed at both 450 and 500 μ M-ATP. The adenylate cyclase activity varied very little over this range of ATP concentrations, and only the data from assays with 500 μ M-ATP are shown (Fig. 3). The adenylate cyclase activity of samples preincubated with ATP alone were calculated at assay ATP concentrations of both 450 and 500 μ M, and varied little over this range (again, only the data calculated for an assay concentration of 500 μ M-ATP are shown).

The data clearly show that ATP alone inhibited the activation

Fig. 2. Activation of platelet adenylate cyclase by p[NH]ppG

(a) Inhibition of p[NH]ppG activation of platelet adenylate cyclase on the inclusion of ATP, cyclic AMP, phosphocreatine and creatine kinase in the membrane preincubation. Two tubes were set up at 30 °C containing 10 μ M-p[NH]ppG and 1 mM-Mg²⁺ either with (■) or without (□) 100 μ M-ATP, 50 μ M-cyclic AMP, 8 mM-phosphocreatine and 0.3 mg of creatine kinase/ml (final concentrations). The preincubations were initiated on the addition of prewarmed membranes. At the indicated times, triplicate samples were removed (containing 15 μ g of membrane protein) to assay the adenylate cyclase activity as described in the Experimental section. This graph is a compilation of data from four separate experiments; the values shown are means of 12 data points $\pm$ S.D. (b) p[NH]ppG activation of platelet adenylate cyclase follows first-order kinetics. The data from (a) were subjected to first-order kinetic analysis. The values used for the maximum rate of adenylate cyclase activity (V_{max}) were those at either the 30 min time point (■, preincubation in the presence of ATP and the other assay components) or the 22.5 min time point (□, control preincubation with only p[NH]ppG and Mg²⁺). The $\ln$ of the difference $V_{max} - v$, where v is the mean adenylate cyclase activity at a particular time point, was plotted against preincubation time for the two sets of conditions and best-fit lines obtained using a least-squares method. The equations of these lines are shown in Table 1.

of the adenylate cyclase complex. Moreover, for the first 5 min of the preincubation time course, this inhibition was indistinguishable from the effects of ATP in conjunction with the other assay components. After this time the two curves diverged. We (Farndale *et al.*, 1987) demonstrated that inclusion of the non-hydrolysable ATP analogue adenosine 5'-[β -imido]triphosphate (p[NH]ppA) in a preincubation with Mg²⁺ and p[NH]ppG did not inhibit activation of adenylate cyclase, and suggested that a terminal phosphate group was essential for obtaining inhibition. The above result is consistent with these observations.

Fig. 3. ATP in the absence of the regenerating system inhibits adenylate cyclase activation by p[NH]ppG

Preincubations were set up at 30 °C with 10 μ M-p[NH]ppG and 1 mM-Mg²⁺ with no further additions (□), with 100 μ M-ATP (●) or with 100 μ M-ATP, 50 μ M-cyclic AMP, 8 mM-phosphocreatine and 0.3 mg of creatine kinase/ml (■). After the indicated periods of preincubation, the adenylate cyclase activity (26 μ g of protein/tube) was assayed for 5 min at 10 mM-Mg²⁺/500 μ M-ATP, as described in the Experimental section. Values shown are means $\pm$ S.E.M. for triplicate determinations.

Adenylate cyclase in human platelet membranes has been shown to be inhibited by ADP in a receptor-mediated manner, with a K_i for ADP of ~ 0.3 μ M (Cooper & Rodbell, 1979). The possibility was considered that the inhibition produced by ATP in both the presence and the absence of the ATP-regenerating system was caused by ADP generated by the hydrolysis of ATP. However, this is somewhat improbable, as platelet P₂ purinergic receptors are unusually specific for ADP and are antagonized by ATP (Cusack *et al.*, 1985). Even in the presence of the regenerating system, up to 10 μ M-ADP could be generated (separation of [α -³²P]-labelled adenine nucleotides by t.l.c.; results not shown). The stable ADP analogue adenosine 5'-[β -thio]-diphosphate (ADP[S]) was added to membrane preincubations with p[NH]ppG and Mg²⁺, in the presence and absence of ATP, cyclic AMP, phosphocreatine and creatine kinase. ADP[S] has also been shown to inhibit platelet adenylate cyclase (K_i for ADP[S] ~ 0.3 μ M) (Cooper & Rodbell, 1979). The results are shown in Fig. 4. ADP[S] caused a slight inhibition of adenylate cyclase activity. However, the inhibition was of the same degree when ADP[S] was included in both the preincubation and the assay or solely in the assay period. In comparison with the large inhibitory effect of ATP in conjunction with the assay components, the effect of ADP[S] appeared to be insignificant. It can therefore be concluded that the inhibitory effect of ATP with or without the ATP-regenerating system was not due to the generation of ADP.

Fig. 3 showed that after 5 min of preincubation the effect of ATP was dependent on the presence or absence of the other assay components. Therefore the effects on the adenylate cyclase activity of the individual assay components, namely phosphocreatine, creatine kinase and cyclic AMP, were investigated (Table 2). Phosphocreatine alone inhibited the adenylate cyclase activity to the same degree as all four assay components together. Creatine kinase on its own did not affect the activation of adenylate cyclase (results not shown). However, a slight increase in the degree of inhibition of adenylate cyclase activity was observed on the inclusion of creatine kinase in conjunction with phosphocreatine. This effect was most noticeable after about

Table 1. p[NH]ppG activation of platelet adenylate cyclase follows first-order kinetics

See the legend to Fig. 2 for details. *r* is the regression coefficient for each line, *k* is the first-order rate constant for the reaction, the slope of the line being $-k$. The *y* intercept of the line is the value of V_{max} . The half-time for the reaction ($t_{1/2}$) is derived from $t_{1/2} = (1/k) \times \ln 2$.

Preincubation conditions	Equation of the line	<i>k</i> (min ⁻¹)	<i>t</i> _{1/2} (min)
p[NH]ppG and Mg ²⁺	$y = 4.743 - 0.1690x$ (<i>r</i> = 1.00)	0.1690	4.101
p[NH]ppG, Mg ²⁺ , ATP, cyclic AMP, phosphocreatine and creatine kinase	$y = 4.4235 - 0.0926x$ (<i>r</i> = 1.00)	0.0926	7.485

Fig. 4. Effect of ADP[S] on the activation of platelet adenylate cyclase by p[NH]ppG

Platelet membranes were preincubated for the specified times at 30 °C in the presence of 10 μM-p[NH]ppG and 1 mM-Mg²⁺ with no other additions (□), with 10 μM-ADP[S] (●), or with 100 μM-ATP, 50 μM-cyclic AMP, 8 mM-phosphocreatine and 0.3 mg of creatine kinase/ml (■). Adenylate cyclase activity (23 μg of membrane protein/tube) either with (●) or without (□, ■) 10 μM-ADP[S] was then assayed for 5 min at 100 μM-ATP as described in the Experimental section. Results are means ± s.e.m. for triplicate determinations.

10 min of preincubation. In general, during this study cyclic AMP was not found to increase the inhibitory effects of phosphocreatine, ATP alone, or ATP in the presence of the assay components. This is in contrast with our previous results (Farndale *et al.*, 1987), which showed a slightly increased inhibition in the presence of cyclic AMP. It is still unclear whether kinase activity is involved with mediating these effects.

Inhibitory effects of the phosphocreatine/creatine kinase ATP-regenerating system on adenylate cyclase activity have previously been recorded (Harwood *et al.*, 1973; Lin *et al.*, 1975; Johnson, 1980), and in all of these cases the inhibition was shown to be due to the phosphocreatine component. We investigated the concentration-dependence of the phosphocreatine-mediated inhibition (Fig. 5). Phosphocreatine only inhibited p[NH]ppG-induced activation of adenylate cyclase when present at concentrations above 1 mM. The inhibition recorded at 10 mM-phosphocreatine was very large, lowering the adenylate cyclase activity by about 50%. The same degree of inhibition was obtained on preincubating membranes with the full complement of the assay components, where the concentration of phosphocreatine used was also 10 mM (results not shown).

Harwood *et al.* (1973) suggested that the inhibition caused by phosphocreatine may have been due to chelation of Mg²⁺.

Fig. 5. Concentration-dependence of inhibition of adenylate cyclase by phosphocreatine

Preincubations of platelet membranes were set up at 30 °C in the presence of 10 μM-p[NH]ppG, 1 mM-Mg²⁺ and increasing concentrations of phosphocreatine. After 15 min, triplicate samples were removed at 6 s intervals for adenylate cyclase assay as described in the Experimental section. Assays contained 23 μg of membrane protein and 10 mM-phosphocreatine. Values are means ± s.e.m. for triplicate determinations.

Table 2. Effects of the individual assay components on adenylate cyclase activity

Platelet membranes (35 μg of protein/tube) were preincubated for the specified times with 10 μM-p[NH]ppG/1 mM-Mg²⁺ with no further additions (Control) or with 100 μM-ATP, 8 mM-phosphocreatine (PCr), 0.3 mg of creatine kinase (CK)/ml and 50 μM-cyclic AMP, alone or in combination. Adenylate cyclase activity was then determined for 5 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.

Conditions	Preincubation time (min)...	Adenylate cyclase activity (pmol/min per mg)			
		4	11	18	25
Control		46 ± 1	89 ± 3	116 ± 3	132 ± 4
ATP + cyclic AMP + PCr + CK		25 ± 2	40 ± 1	55 ± 1	64 ± 3
PCr		16 ± 7	39 ± 1	56 ± 1	63 ± 1
PCr + cyclic AMP		18 ± 4	37 ± 1	51 ± 1	61 ± 1
PCr + CK		17 ± 1	29 ± 1	47 ± 1	58 ± 2
PCr + CK + cyclic AMP		18 ± 1	33 ± 1	50 ± 3	63 ± 4

Although phosphocreatine had a weak affinity for Mg²⁺ (the calculated apparent stability constant at pH 8.0 is 39.8), we considered the possibility that under the preincubation conditions

Fig. 6. Inhibition of adenylate cyclase by phosphocreatine is not due solely to the chelation of free Mg^{2+}

Tubes were set up at 30 °C containing 10 μ M-p[NH]ppG at various Mg^{2+} concentrations from 0.5 to 10 mM in the absence (□) or the presence (■) of 8 mM-phosphocreatine (final concentrations). Preincubations were initiated on the addition of prewarmed membranes. The actual free Mg^{2+} concentration of preincubations in the presence of phosphocreatine was calculated according to Fabiato & Fabiato (1979), and these corrected values have been used. After 5 min of preincubation, triplicate samples containing 26 μ g of membrane protein were withdrawn at 6 s intervals. The adenylate cyclase activity was determined at 10 mM- Mg^{2+} as described in the Experimental section. Results are means $\pm$ S.E.M. for triplicate assays.

used in these experiments phosphocreatine may have decreased the concentration of free Mg^{2+} . This would be particularly important, as we (Farndale *et al.*, 1987) demonstrated previously that the activation by p[NH]ppG in these membranes is particularly sensitive to the Mg^{2+} concentration in the preincubation. Fig. 6 shows the result of an experiment where membranes were preincubated for 5 min with p[NH]ppG at various Mg^{2+} concentrations from 0.5 to 10 mM, with and without 8 mM-phosphocreatine. The adenylate cyclase activity of all the samples was assayed at 10 mM- Mg^{2+} . The actual free Mg^{2+} concentrations during a preincubation with 8 mM-phosphocreatine, having allowed for chelation by the phosphocreatine, were calculated according to Fabiato & Fabiato (1979), and these corrected values are plotted in Fig. 6. The data demonstrate that the inhibitory effect of phosphocreatine is not due solely to the chelation and therefore decrease in the concentration of free Mg^{2+} ions. Inhibition was most marked over the concentration range 0.5–2 mM- Mg^{2+} . The Mg^{2+} concentration used routinely during this study was 1 mM, which falls within this range. Inhibition by phosphocreatine was also evident after longer periods of preincubation (results not shown).

In the absence of an ATP-regenerating system, ATP is degraded by platelet membranes (results not shown). Therefore, to examine effects of ATP over a relatively longer period of time than was possible in Fig. 3, inclusion of the regenerating system becomes necessary. The possibility was considered that the inhibitory effect of ATP and the other assay components might vary as the preincubation time course progressed. Membrane preincubations were set up with p[NH]ppG and Mg^{2+} to which ATP, cyclic AMP, phosphocreatine and creatine kinase were added at various stages of the time course, and samples were removed to assay the adenylate cyclase activity. The results of experiments in which these additions were made after 1 or 2 min of preincubation and 6 or 9 min of preincubation are shown in Figs. 7(a) and 7(b) respectively. These experiments show that

Fig. 7. Activation by p[NH]ppG is inhibited by the delayed addition of the assay components after the start of the preincubation

(a) Platelet membranes (43 μ g of protein/tube) were preincubated in the presence of 10 μ M-p[NH]ppG and 1 mM- Mg^{2+} either without (□) or with 100 μ M-ATP, 50 μ M-cyclic AMP, 8 mM-phosphocreatine and 0.3 mg of creatine kinase/ml (all final concentrations). These additions were either present from the start of the preincubation (■, 0 min) or otherwise added 1 min (●) or 2 min (○) after the start, in a volume that was 5% of the final preincubation volume (the necessary adjustments being made to the p[NH]ppG and Mg^{2+} concentrations). At the indicated times, triplicate samples were withdrawn at 6 s intervals for adenylate cyclase assay as described in the Experimental section. (b) Two tubes were set up at 30 °C containing 10 μ M-p[NH]ppG and 1 mM- Mg^{2+} without (□), or with (■) 100 μ M-ATP, 50 μ M-cyclic AMP, 8 mM-phosphocreatine and 0.3 mg of creatine kinase/ml (all final concentrations). Preincubations were initiated by pipetting in prewarmed membranes. The preincubation in the absence of the assay components was subsequently subdivided to make three parallel preincubations. At 6 min (●) and 9 min (○) after the start, the same additions (ATP, cyclic AMP and the ATP-regenerating system) were added to two out of the three preincubations, in a volume that was 5% of the final preincubation volume. Adenylate cyclase activity (22 or 23 μ g of membrane protein/tube) was determined as described in the Experimental section. Results are means $\pm$ S.E.M. for triplicate assays.

inhibition could be observed at both early and later time points during the activation process. The inhibition was rapid, and drastically decreased the speed of the activation. The maximum activity was also less than that obtained when the membranes were maintained in the presence of p[NH]ppG and Mg^{2+} alone.

We next considered the possibility that the apparent maximal activation of adenylate cyclase recorded after prolonged preincubation presented an equilibrium position where the stimulation due to p[NH]ppG was opposed by the inhibitory effect of

Fig. 8. Activation of platelet adenylate cyclase by p[NH]ppG is reversed by the assay components

Two preincubations of platelet membranes (0.76 mg of protein/ml) were set up at 30 °C in the presence of 10 μ M-p[NH]ppG and 1 mM-Mg²⁺ with and without 100 μ M-ATP, 50 μ M-cyclic AMP, 8 mM-phosphocreatine and creatine kinase (0.3 mg/ml). After 25 min, membranes were subsequently diluted into a second preincubation (0.38 mg protein/ml). Those initially preincubated with only p[NH]ppG and Mg²⁺ were diluted into p[NH]ppG and Mg²⁺ in the presence (●, B) or absence (□, A) of ATP, cyclic AMP, phosphocreatine and creatine kinase (concentrations as above). Membranes from the initial preincubation in the presence of p[NH]ppG, Mg²⁺ and the assay components were diluted into a solution of the same (■, C). At the indicated times, triplicate samples (containing 19 μ g of membrane protein) were withdrawn at 6 s intervals for adenylate cyclase assay as described in the Experimental section. Values are means $\pm$ S.E.M. for triplicate determinations.

Fig. 9. Effect of GDP[S] on the activation of platelet adenylate cyclase by p[NH]ppG

A set of six preincubations were set up at 30 °C and initiated on the addition of warmed preincubation mix to an equal volume of prewarmed membranes, giving final concentrations of 10 μ M-p[NH]ppG and 1 mM-Mg²⁺. After 0, 3, 7, 12 or 18 min of preincubation, GDP[S] (final concentration 100 μ M) was added in a small volume (1 % of the preincubation volume) to five of the preincubations. Samples of 50 μ l containing 36 μ g of membrane protein were removed at regular intervals to assay the adenylate cyclase activity as described in the Experimental section. Activities shown with (■) or without (□) GDP[S] are means $\pm$ S.E.M. for triplicate determinations.

membranes were maintained throughout with p[NH]ppG, Mg²⁺, ATP, cyclic AMP and the ATP-regenerating system (curve C). At various time intervals, samples were removed to assay the adenylate cyclase activity. The data show that a slow decline in adenylate cyclase activity occurred on transferring membranes from conditions without to conditions with ATP, cyclic AMP and the ATP-regenerating system (curve B). This demonstrates that the components of the assay system were capable not only of slowing down the activation obtained with p[NH]ppG and Mg²⁺, but also of reversing the process. The maximally activated state of adenylate cyclase was indeed still sensitive to inhibition. The reversal under the conditions for B relative to those for A is likely to be an understatement of the effects of ATP, cyclic AMP and the ATP-regeneration system because the control curve (A) also declined with time, but to a significantly lesser extent. It is well known that the catalytic unit undergoes thermal inactivation and that ATP can be used to protect against this (Ross *et al.*, 1978). In curve C, where ATP, cyclic AMP, phosphocreatine and creatine kinase were present throughout, no such inactivation or reversal occurred, suggesting that only under these conditions was a true equilibrium between activation and inhibition achieved.

The results shown in Fig. 1 demonstrate that the activation induced by p[NH]ppG corresponds to increasing activation of G_s. To investigate this further, a series of parallel incubations with p[NH]ppG and Mg²⁺ were set up to which the stable GDP analogue GDP[S] (100 μ M) was added at various time points after the start of the experiment. This tested whether the activation could be inhibited by GDP[S] and whether sensitivity to the GDP analogue persisted during the process of activation (Fig. 9). GDP[S] blocked the activation of adenylate cyclase by p[NH]ppG completely and immediately upon addition. When GDP[S] was present from the start of the preincubation, activation of adenylate cyclase was blocked. When GDP[S] was added at an intermediate stage of the activation process, further activation was blocked and the adenylate cyclase activity remained approximately constant for the remainder of the experiment. The inhibition observed was not an effect of GDP[S] occurring during the assay period, as only a slight decrease in adenylate cyclase activity occurred when GDP[S] was present solely during the 5 min assay. This lack of effect was maintained throughout preincubation (results not shown).

Fig. 10(a) shows the concentration-dependence of inhibition by GDP[S]. A series of parallel preincubations was set up containing increasing concentrations of GDP[S]. Preincubations contained p[NH]ppG and Mg²⁺, with and without the ATP, cyclic AMP, phosphocreatine and creatine kinase. At various time intervals, samples were removed to assay the adenylate cyclase activity, at the same concentration of GDP[S] as was present in the preincubation. The data show that both types of preincubation responded similarly to increasing concentrations of GDP[S]. A maximal concentration of GDP[S] (100 μ M) completely blocked the activation of adenylate cyclase; the activity was no greater than basal activity measured on pipetting membranes directly into an assay at this concentration of GDP[S] (results not shown). At submaximal concentrations of GDP[S] activation of adenylate cyclase did occur, the degree of activation being closely related to the GDP[S] concentration. Basal levels of adenylate cyclase activity decreased very slightly as the GDP[S] concentration present during the assay increased (results not shown). Fig. 10(b) is a re-plot of the data from Fig. 10(a). The adenylate cyclase activity for each GDP[S] concentration is expressed as a percentage of the activity measured when no GDP[S] was added to the preincubation. Therefore, with increasing concentrations of GDP[S], the percentage of activity remaining declined.

the other assay components. Fig. 8 shows the results of an experiment where membranes were preincubated for 25 min with p[NH]ppG and Mg²⁺, after which time the activation was presumed to be maximal. The membranes were then transferred to a second preincubation, also with p[NH]ppG and Mg²⁺ but in the presence (curve B) or absence (curve A) of ATP, cyclic AMP, phosphocreatine and creatine kinase. In a parallel incubation,

Fig. 10. Inhibition of adenylate cyclase activity by GDP[S]

(a) Concentration-dependence of GDP[S] inhibition. A series of preincubations with 10 μM-p[NH]ppG and 1 mM-Mg²⁺ in the absence (□) and presence (■) of 100 μM-ATP, 50 μM-cyclic AMP, 8 mM-phosphocreatine and creatine kinase (0.3 mg/ml) were set up at 30 °C. In addition, increasing concentrations of GDP[S] were included. After 20 min, triplicate samples containing 26 μg of membrane protein were removed at 6 s intervals to assay adenylate cyclase. The same final concentration of GDP[S] as had been present in a preincubation was maintained in the assay. Adenylate cyclase activity was determined as described in the Experimental section. Results are means ± S.E.M. for triplicate assays. (b) The inhibitory effect of GDP[S] on adenylate cyclase activation is the same under both sets of preincubation conditions. This is a re-plot of data from (a). The adenylate cyclase activity at a particular concentration of GDP[S] is plotted relative to the activity when no GDP[S] was included in the preincubation. Results are expressed as a percentage of the activity observed without GDP[S].

Both types of preincubation were equally sensitive to inhibition by increasing concentrations of GDP[S]; the curves are essentially overlapping. Therefore the two types of inhibition of platelet adenylate cyclase observed during this study, i.e. inhibition caused by GDP[S] and inhibition caused by ATP and the other assay components, are due to two different mechanisms.

General discussion

The data presented here provide evidence that a slow change in the activity of G_s is the mechanism for the activation produced by p[NH]ppG and Mg²⁺. Results from experiments with forskolin demonstrated that maximal activation of the adenylate cyclase complex was observed only after a prolonged period of in-

cubation with p[NH]ppG and Mg²⁺. Although forskolin markedly enhanced adenylate cyclase activity, presumably by activating the catalytic unit, this did not override the stimulatory effect of the interaction between the catalytic unit and G_s (Fig. 1). This suggests that the locus of the slow time-dependent increase in adenylate cyclase activity is distinct from the forskolin-binding site on the catalytic unit. The addition of 100 μM-GDP[S] blocked further activation with p[NH]ppG and Mg²⁺ at all stages of the preincubation time course (Fig. 9). Inhibition was immediate and the adenylate cyclase activity was held constant for the continuation of the experiment. This provides further evidence that the process underlying the activation is a change in activity of G_s. A decrease in activity of G_i can be excluded, as the presence of either ADP[S] (Fig. 4) or adrenaline (Farndale *et al.*, 1987) in the incubation with p[NH]ppG and Mg²⁺ did not block the activation process. Both are agonists for the G_i-linked pathway.

Activation of platelet adenylate cyclase by p[NH]ppG and Mg²⁺ was inhibited by ATP in both the presence and the absence of the ATP-regenerating system. The inclusion of ATP at the start of a preincubation slowed down the progress of activation (Figs. 2a and 3). Activation was also slowed when ATP in the presence of cyclic AMP, phosphocreatine and creatine kinase was added some time after the start of the experiment (Figs. 7a and 7b). A state of maximal activation of the adenylate cyclase complex obtained after a prolonged preincubation with p[NH]ppG and Mg²⁺ was still sensitive to ATP and the other assay components. On addition, they caused a slow decline in activity (Fig. 8). It is possible that the lower maximal activity obtained in the presence of the assay components represents an equilibrium position where activatory and inhibitory forces are temporarily in balance. These results demonstrate that the mechanism of activation of platelet adenylate cyclase by p[NH]ppG is highly sensitive to the experimental conditions.

The high degree of sensitivity of the activation process is revealed by the kinetic data. In both the presence and the absence of ATP and the other assay components, the activation followed first-order kinetics (Figs. 2a and 2b). The first-order rate constants were respectively 0.09 min⁻¹ and 0.17 min⁻¹. The *t*_{1/2} values for the processes were approx. 7.5 min in the presence of the assay components and 4 min in their absence, showing that the activation was slowed to about half its previous rate. These catalytic constants are very small; most single-step enzyme-catalysed reactions occur within the millisecond time scale and have correspondingly much greater catalytic constants. However, these kinetics describe a complex sequence of events corresponding to an increase in the degree of activation of G_s taking place in what is an already activated adenylate cyclase system. Due to the extreme slowness of this activation process, especially in the presence of ATP, cyclic AMP, phosphocreatine and creatine kinase, the conditions under which the vast preponderance of previous studies have been conducted, it is possible that similar effects on adenylate cyclase in other tissues have not been recognized. This may explain why many authors have concluded that activation of adenylate cyclase by non-hydrolysable GTP analogues results in a constant rate of production of cyclic AMP (Rodbell *et al.*, 1975; Iyengar & Birnbaumer, 1981; Jakobs *et al.*, 1983; Farndale *et al.*, 1987), contrasting with the slowly changing rate of cyclic AMP generation that we have observed.

During this study, it became evident that a constituent of the ATP regenerating system, i.e. phosphocreatine, at concentrations above 1 mM, inhibited adenylate cyclase activity to a similar degree as did the full complement of assay components (Table 2 and Fig. 5). Similar inhibitory effects have also been observed in studies on rat liver adenylate cyclase (Harwood *et al.*, 1973; Lin *et al.*, 1975; Johnson, 1980). The inhibition was not found to be

due to the chelation of Mg^{2+} ions (Fig. 6), a possible mechanism of inhibition suggested by Lin *et al.* (1975).

Possible mechanisms for the inhibition by phosphocreatine include the generation of ATP from endogenous ADP and membrane-associated creatine kinase. Platelets store both ADP and ATP in dense secretory granules (Zucker & Nachmias, 1985). The method of platelet membrane preparation that we and many others employ produces a crude plasma membrane fraction which may well be contaminated with adenine nucleotides. The K_m of creatine kinase for phosphocreatine is 5 mM at pH 7.0 (Kuby *et al.*, 1954), which is consistent with the observed concentration-dependence of inhibition. The data in Table 2 show a small increase in the inhibition of adenylate cyclase activity when exogenous creatine kinase was added in conjunction with phosphocreatine. This suggests that the mechanism of the inhibition produced by phosphocreatine may involve creatine kinase. However, the inhibitory effect of phosphocreatine may be mediated quite differently. The possibility that phosphocreatine acts directly as a phosphate donor for protein phosphorylation cannot be excluded.

We considered the possibility that ATP-dependent inhibition of adenylate cyclase activity (Figs. 2a and 3) was mediated via guanine nucleotides present in the membrane preparation or as contaminants of ATP, phosphocreatine, creatine kinase or p[NH]ppG. Generation of GDP and/or GTP from GMP and GDP might lead to competition with p[NH]ppG, thereby blocking adenylate cyclase activation. We (Farndale *et al.*, 1987) found no evidence for a possible inhibitory effect of GTP: the addition of 1 μ M-GTP to a preincubation had no effect on the activation of adenylate cyclase by p[NH]ppG. It is unlikely that this concentration of GTP would be obtained from a source of contamination. The data presented in Figs. 10(a) and 10(b) show that the inhibitory effect of ATP is not in fact due to GDP production. Fig. 10(b) demonstrates that membrane pre-incubations in both the presence and the absence of the full ATP-regenerating system had identical sensitivity to inhibition by GDP[S]. The preincubation with ATP, cyclic AMP, phosphocreatine and creatine kinase contained all the above possible sources of contaminating guanine nucleotides, and yet showed the same sensitivity to GDP[S] as did the preincubation without any of these additions.

The experimental conditions of an adenylate cyclase assay are set up to maintain the ATP concentration during the assay to prevent a decline in the rate of turnover of the catalytic unit of adenylate cyclase. These conditions also provide an environment which supports the activity of endogenous protein kinases present in the membrane preparation (results not shown). The above results showing a decreased rate of activation of adenylate cyclase in the presence of ATP are consistent with the idea that activation may be due to a dephosphorylation event, and the inhibition observed with ATP is a result of protein phosphorylation by the action of a membrane-associated protein kinase. We (Farndale *et al.*, 1987) have previously suggested from our results that the kinase involved may be cyclic AMP-dependent protein kinase A. The lack of effect of cyclic AMP found in the present study is in contrast to the slight enhancement of ATP-induced inhibition previously observed (Farndale *et al.*, 1987). However, the adenylate cyclase activity observed under these experimental conditions may generate sufficient cyclic AMP to rapidly activate an endogenous protein kinase A. The possible substrates for protein phosphorylation include G_s , the catalytic unit of adenylate cyclase, or a regulatory protein which interacts with the adenylate cyclase complex.

In general, platelet activation is known to be closely involved with cycles of protein phosphorylation and dephosphorylation; for example, myosin light chain, actin-binding protein and a

47 kDa protein are known to be phosphorylated during platelet activation (see Gerrard *et al.*, 1987). Activation of the phospholipase C-linked signal pathway and pathways producing cyclic nucleotides all cause the activation of a variety of protein kinases. The identification of protein phosphatases is less advanced in platelets, but a calcium/calmodulin-regulated phosphatase has been identified in human platelet extracts (Tallant & Wallace, 1985). The data presented here suggest that such events may have an important role in the regulation of platelet adenylate cyclase.

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