The role of G_i and the membrane-fluidizing agent benzyl alcohol in modulating the hysteretic activation of human platelet adenylate cyclase by guanylyl 5'-imidodiphosphate

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The non-hydrolysable GTP analogue guanylyl 5'-imidodiphosphate (p[NH]ppG) elicited a profound increase in the adenylate cyclase activity of human platelets. This occurred after a well-defined lag period of around 6 min, whereupon an enhanced steady-state rate was evident. The duration of the lag period was unchanged over a range of concentrations of p[NH]ppG which gave very different steady-state rates of adenylate cyclase activity. Prior activation of the stimulatory G-protein G_s by cholera-toxin pre-treatment abolished the lag period and elicited a small increase in the steady-state rate. Manipulating function of the inhibitory G-protein G_i also led to profound changes in the lag periods. Thus marked decreases in the lag were seen (~ 70 – 81%) when G_i function was ablated through pre-treatment of platelet membranes with pertussis toxin, or by using elevated (25 mM) Mg^{2+} levels in the assay, or when Mg^{2+} was replaced by 5 mM Mn^{2+} in the assay. In contrast with this, potentiation of G_i function led to an increase in the lag period, as seen under conditions of agonist occupancy of

inhibitory α_2 -adrenoceptors (increase $\sim 74\%$) or with the addition of 100 mM NaCl to the assays (increase $\sim 44\%$). The local anaesthetic and membrane-fluidizing agent benzyl alcohol elicited both a profound decrease (around 70% at 80 mM) in the p[NH]ppG-induced lag period and a marked augmentation (around 5-fold) in the steady-state adenylate cyclase activity. When adenylate cyclase assays were done at 35 °C instead of 25 °C, then the lag period for activation by p[NH]ppG was decreased by around 33% and the steady-state rate increased by around 3-fold. At 35 °C, the addition of benzyl alcohol led to the apparent abolition of the lag period for p[NH]ppG activation of adenylate cyclase and amplified the steady-state rate by only around 2.2-fold. It is shown that G_i plays a fundamental role in determining the rate of activation of G_s . The proposal is formulated that such an action may be mediated through the release of $\beta\gamma$ -subunits. Thus $\beta\gamma$ -subunit dissociation is proposed as providing the rate-limiting step in G_i activation.

INTRODUCTION

Adenylate cyclase is under the dual control of both stimulatory and inhibitory receptors, whose actions are mediated by distinct G-proteins called G_s and G_i respectively. Such G-proteins are heterotrimeric species consisting of distinct GTP-binding α -subunits, together with β - and γ -subunits, which appear to be identical for both G_i and G_s species. Agonist occupancy of appropriate receptors elicits a conformational change, allowing the complex to couple to an appropriate G-protein which then binds GTP. This leads to the dissociation of the complex, releasing an activated GTP-bound α -subunit, which can then interact with adenylate cyclase (see Gilman, 1987; Birnbaumer et al., 1990).

Three forms of G_i have now been identified, all of which are subject to ADP-ribosylation by pertussis toxin, but, whereas G_{i-2} appears to be universally expressed in mammalian cells, selective expression of G_{i-1} or G_{i-3} is seen (Birnbaumer et al., 1990). Thus in platelets only G_{i-2} and G_{i-3} are expressed (Livingstone et al., 1991). It is a matter of some debate (Birnbaumer et al., 1990) as to whether only certain or all of these G_i forms can mediate the inhibition of adenylate cyclase. However, a number of investigators, employing independent approaches, have shown that at least G_{i-2} can effect such an action (Bushfield et al., 1990; McKenzie & Milligan, 1990; Simonds et al., 1990; Senogles et al., 1990). Similarly, the mechanism whereby G_i elicits inhibition of adenylate cyclase has yet to be fully elucidated (Birnbaumer et

al., 1990). However, contributions to inhibition are likely to arise from two routes, namely the release of $\beta\gamma$ -subunits from G_i , which can be expected to inhibit the dissociation and activation of G_s , and also a direct inhibitory effect of GTP-bound G_i α -subunit ($\alpha-G_i$) on the catalytic unit of adenylate cyclase itself (Birnbaumer et al., 1990). Indeed, using the local anaesthetic benzyl alcohol, we have attempted to determine the relative importance of both of these routes to the inhibitory regulation of adenylate cyclase in human platelets (Spence and Houslay, 1990). This compound increases the fluidity of platelet and other biological membranes and can potentiate the action of stimulatory receptors on adenylate cyclase activity by increasing the conformational flexibility of the proteins involved (Dipple and Houslay, 1978; Gordon et al., 1980, 1983; Houslay and Gordon, 1983; Houslay, 1986). However, it is capable of eliciting selective effects on the G_i -mediated inhibitory regulation of adenylate cyclase (Spence and Houslay, 1990) by partially attenuating G_i function when adenylate cyclase is coupled to stimulatory receptors, while not affecting direct G_i -mediated inhibition of the catalytic unit of this enzyme. This discriminatory action has been attributed (Spence and Houslay, 1990) to the ability of benzyl alcohol to disrupt $\beta\gamma$ -subunit interaction with G-protein α -subunits, hence destabilizing the inactive holomeric state.

G-proteins can be activated in the absence of occupied stimulatory receptors (Gilman, 1987; Birnbaumer et al., 1990) by non-hydrolysable GTP analogues such as guanylyl 5'-imidodi-

Abbreviations used: G-proteins, guanine nucleotide regulatory proteins; G_s , stimulatory G-protein regulating adenylate cyclase; G_i , inhibitory G-protein regulating adenylate cyclase; p[NH]ppG, guanylyl 5'-imidodiphosphate.

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phosphate (p[NH]ppG). A number of studies, done primarily on membranes from liver and neuroblastoma (Salomon et al., 1975; Blume and Foster, 1976; Levinson and Blume, 1977; Iyengar and Birnbaumer, 1979; Birnbaumer et al., 1980; Iyengar et al., 1980; Iyengar and Birnbaumer, 1981; Iyengar, 1981), but also noted in platelets (Steer and Wood, 1979; Farndale et al., 1987), have shown that p[NH]ppG leads to a profound stimulation of adenylate cyclase activity, but that this occurs after a well-defined lag period. This lag period has been shown not to be due to a slow rate of exchange of the analogue with G-protein-bound GDP (Birnbaumer et al., 1980; Iyengar and Birnbaumer, 1981; Iyengar, 1981) or to any property of the catalytic unit of adenylate cyclase itself (Iyengar, 1981). Rather, it has been suggested to reflect a hysteretic event whereupon the α -subunit of the stimulatory G-protein G_s (α - G_s) adopts an activated conformation (Birnbaumer et al., 1980; Iyengar and Birnbaumer, 1981). This hysteretic phenomenon could presumably be attributed either to the release of $\beta\gamma$ -subunits from p[NH]ppG-bound G_s or to an activating transition in p[NH]ppG-bound α - G_s itself. The ensuing steady-state rate reflects adenylate cyclase which has been constitutively activated due to the fact that p[NH]ppG-bound α - G_s cannot be de-activated through GTPase activity. Thus the ability of p[NH]ppG, in the absence of agonist-occupied receptors coupled to G_s , to elicit activation of G_s , and hence adenylate cyclase, in 'slow motion', provides us with a useful tool to determine factors which influence the rate of activation of G_s in a biological membrane. Here we exploit this phenomenon to show that, in human platelets, the rate of activation of G_s can be determined by input from G_i and also influenced markedly through the action of the local anaesthetic and membrane-fluidizing agent benzyl alcohol.

MATERIALS AND METHODS

Human platelet membranes were prepared as described previously (MacIntyre and Pollock, 1983; Houslay et al., 1986a,b), from platelet-rich plasma (Blood Transfusion Service, Law Hospital, Carlisle, Scotland, U.K.) and stored frozen at -80°C until required. Protein content was estimated by the method of Lowry et al. (1951) as modified by Peterson (1977), with BSA as standard.

Adenylate cyclase activity was measured by an adaptation of the method of Salomon et al. (1974). Briefly, platelet membranes (20–100 μg of protein) were incubated in a reaction mixture consisting of 50 μM ATP, 5 mM phosphocreatine, 0.4 mg/ml creatine kinase, 2 mg/ml BSA and 0.1 mM cyclic AMP in a buffer of 50 mM triethanolamine, pH 7.4, containing 2 mM MgCl_2 , 0.1 mM EGTA, 1 mM isobutylmethylxanthine and 1 mM dithiothreitol. [α - ^{32}P]ATP was added to a concentration of 2 μCi /tube and incubations were continued for 0–20 min. Reaction tubes lacking ATP, [α - ^{32}P]ATP, p[NH]ppG and agonists were warmed to assay temperature (25°C) by incubation for 2 min, and then the reaction was initiated by their addition as a 'cocktail'. In all cases the volume of the addition used to start the reaction was kept constant and elicited $<2^\circ\text{C}$ change in temperature of the reaction mixture. In preincubation experiments samples were incubated at 25°C for 5 or 10 min (as indicated) before addition of the labelled ATP. Final volumes were 100 μl in all cases. Incubations were terminated by the addition of a 'stopping solution' containing 2% SDS, 40 mM ATP and 1.4 mM cyclic AMP, at pH 7.5. Cyclic AMP levels were determined as described by Salomon et al. (1974), by using successive chromatography on Dowex and alumina columns.

Mono-ADP-ribosylations were performed on platelet membranes as described previously by us in detail (Houslay et al.,

1986a,b) by using pre-activated (exposed to an equal volume of 40 mM dithiothreitol) bacterial toxins, and a ribosylation cocktail comprising 1 mM GTP, 2 mM NAD^+ , 30 mM thymidine and 100 mM potassium phosphate, pH 7.4. In pertussis-toxin-mediated ribosylations 2 mM ATP was also present in this cocktail. Platelet membranes were incubated with this mixture at 30°C for 10–30 min, followed by centrifugation at 14000 g for 15 min at 4°C . Pelleted membranes were washed twice and finally resuspended in 10 mM Tris/HCl, pH 7.4.

RESULTS AND DISCUSSION

A number of investigators (Steer and Wood, 1979; Birnbaumer et al., 1980; Iyengar and Birnbaumer, 1981; Iyengar, 1981; Farndale et al., 1987) have demonstrated that the activation of adenylate cyclase by p[NH]ppG occurs with a well-defined lag period, after which time a new, increased, steady-state rate is achieved. The ability of p[NH]ppG to elicit the activation of G_s , and hence adenylate cyclase, with such a discernible hysteretic event thus provides us with a useful tool to determine factors which influence the rate of activation of G_s in a biological membrane. The most detailed analysis of this hysteretic phenomenon has been done in rat liver plasma membranes (Birnbaumer et al., 1980; Iyengar and Birnbaumer, 1981; Iyengar, 1981), but such hysteresis has also been noted in human platelets (Steer and Wood, 1979; Farndale et al., 1987), as we confirm here (Figure 1). Thus treatment of human platelet membranes with 100 μM p[NH]ppG, together with 5 mM MgCl_2 , caused the marked

Figure 1 Hysteretic activation of adenylate cyclase by p[NH]ppG

Data shows a typical progress curve to demonstrate the lag before steady-state activity is reached for adenylate cyclase assays done in the presence of 100 μM p[NH]ppG alone (●), as well as the shifts in lag observed in the presence of 50 mM benzyl alcohol (□) and with α_2 -adrenergic inhibitory input using 100 μM adrenaline together with 10 μM propranolol (○). Iterative regression analyses were done to gauge the linear segments over which steady-state rates were achieved, and hence, by extrapolation, the lag period from its conjunction with the abscissa (Houslay and Palmer, 1978). Activity is expressed in nmol of cyclic AMP produced/mg of membrane protein. For clarity, in the Figure the cyclic AMP produced in the presence of benzyl alcohol has been decreased to half the value actually produced.

Table 1 Activation of adenylate cyclase by p[NH]ppG

In each experimental set, lag periods were compared with those exhibited by adenylate cyclase activity assayed in the presence of 100 μ M p[NH]ppG at 25 °C (5.7 min set as 100%). The magnitude of this lag time was 5.7 ± 0.6 min (S.D., $n = 6$ different membrane preparations). Basal adenylate cyclase activity was 118 ± 29 pmol of cyclic AMP formed/min per mg of membrane protein at 25 °C, with no observable lag over an apparently linear time course followed for 30 min. Where indicated, membranes were pre-treated with thiol-activated cholera toxin (CT) or pertussis toxin (PT) as described in the Materials and methods section. Each experimental set was done three times using different membrane preparations, with adenylate cyclase assays in triplicate, and with errors given as S.D. Abbreviation: n.e., not evident, i.e. no observable lag (< 0.5 min). * Activities expressed as a percentage of that activity observed in the presence of 100 μ M p[NH]ppG + 5 mM MgCl₂ and assayed at 25 °C.

Ligand challenge	Lag period (min)	Relative lag period (%)	Steady-state rate (pmol/min per mg of protein)	Steady-state rate (%)
1 mM p[NH]ppG + 5 mM MgCl ₂	5.9 ± 0.4	104	970 ± 15	99
100 μ M p[NH]ppG + 5 mM MgCl ₂	5.7 ± 0.6	(100)	980 ± 22	(100)
1 μ M p[NH]ppG + 5 mM MgCl ₂	5.9 ± 0.5	104	402 ± 49	41
0.1 μ M p[NH]ppG + 5 mM MgCl ₂	6.2 ± 0.6	109	333 ± 29	34
100 μ M p[NH]ppG + 25 mM MgCl ₂	1.0 ± 0.3	18	1009 ± 78	103
100 μ M p[NH]ppG + 5 mM MnCl ₂	1.8 ± 0.6	32	1000 ± 115	102
100 μ M p[NH]ppG + 5 mM MgCl ₂ + 100 mM NaCl	8.2 ± 1.0	144	862 ± 69	88
CT-pre-treated membranes	n.e. (< 0.5)	0	1274 ± 147	130
PT-pre-treated membranes	1.1 ± 0.4	19	1019 ± 78	104
+ 100 μ M adrenaline + 10 μ M propranolol	9.9 ± 0.8	174	1038 ± 98	106

activation of adenylate cyclase after a well-defined lag of around 6 min (Table 1). Studies done on rat liver indicated that the lag was not due to the slow exchange of GDP for p[NH]ppG on G_s (Iyengar et al., 1979, 1980; Iyengar and Birnbaumer, 1979; Birnbaumer et al., 1980), which would be consistent with our observations (Table 1) that the lag time was unchanged when using a range of p[NH]ppG concentrations which each elicited very different steady-state rates. Indeed, challenge with 100 μ M p[NH]ppG gave maximal activation, yet still showed a clear lag which was unaltered even when using a 10-fold excess (1 mM) of p[NH]ppG (Table 1). The source of the hysteretic event has been suggested to lie at the level of G_s, and, certainly, purified G_s can undergo a time-dependent dissociation phenomenon (Iyengar, 1981). In accord with this, Farndale et al. (1987) have shown that pre-incubation of platelet membranes with p[NH]ppG, before the start of the adenylate cyclase assay, led to the abolition of the lag phase, and we show here that the prior dissociation and activation of G_s, with thiol-preactivated cholera toxin, led to the abolition of p[NH]ppG-induced hysteresis, as evidenced by the loss of the lag period (Table 1). What remains unclear is whether the observed hysteresis is determined primarily by the (slow) rate of dissociation by $\beta\gamma$ -subunits from p[NH]ppG-bound α -G_s or by the (slow) adoption of an active conformation by p[NH]ppG-bound α -G_s.

Analyses done to define the basis of this hysteretic phenomenon, however, pre-dated the discovery of inhibitory input through the G_i system. This is of particular importance, as input from both G_s and G_i can be expected to define the rate of activation of adenylate cyclase, the degree of activation elicited by stimulatory receptors and the level of basal enzyme activity seen at resting GTP levels of around 600 μ M in many cells (Houslay, 1986). Indeed, G_i is poised to play a pivotal role in determining the rate of G_s activation, as guanine nucleotides will bind to G_i at a faster rate than they do to G_s (Carty et al., 1990; Graziano et al., 1989; Freissmuth et al., 1989). The consequence of this is that the prior activation and dissociation of G_i will cause the release of $\beta\gamma$ -subunits which, through Mass Action, will inhibit G_s dissociation. Thus, if the release of $\beta\gamma$ -subunits from G_s underlies, or contributes to, the hysteretic phenomenon,

then the activation of G_i will serve to increase the time taken for adenylate cyclase to reach its steady-state rate. We have investigated this using a number of different approaches. Firstly, we analysed the effect of agonist occupancy of the inhibitory α_2 -adrenoceptor on the lag time (Table 1). For, as the stimulatory effect of p[NH]ppG is enhanced in the presence of agonists for G_s-linked receptors (Gilman, 1987; Birnbaumer et al., 1990), then the same is likely to be true for the functioning of G_i in the presence of agonists for inhibitory receptors. Indeed, we see here (Table 1) that agonist occupancy of the inhibitory α_2 -adrenoceptor led to a marked increase (74%) in the lag time, consistent with inhibitory receptor occupancy augmenting the rapid dissociation and activation of G_i, which would then attenuate G_s dissociation and activation (Table 1). Secondly, Jakobs (1979) has shown that high concentrations of NaCl can potentiate G_i action, and here we exploit this to demonstrate that addition of 100 mM NaCl to assays markedly extended ($\sim 44\%$) the lag time (Table 1). In these instances, however, the steady-state rate achieved with p[NH]ppG was little changed (Table 1). This is likely to be because maximally effective concentrations of p[NH]ppG (100 μ M in Table 1; but also with 1 mM) will eventually elicit the complete dissociation of G_s and hence the constitutive activation of adenylate cyclase. This is consistent with reconstitution studies done with purified G-proteins (Gilman, 1987) and may be because either p[NH]ppG-bound α -G_s provides an over-riding influence on adenylate cyclase activity or because it binds to adenylate cyclase at a common site, but with higher affinity, than does p[NH]ppG-bound α -G_i.

A third approach employed pertussis toxin to attenuate the functioning of G_i. This leads to the loss of α_2 -adrenoceptor-agonist-stimulated GTPase activity and inhibition of adenylate cyclase in platelets (Houslay et al., 1986a,b; Gawler and Houslay, 1987). By causing the ADP-ribosylation of the C-terminal cysteine of α -G_i, it elicits uncoupling from the inhibitory receptor (Birnbaumer et al., 1990) and can also ablate the guanine-nucleotide-triggered inhibition of adenylate cyclase (Katada et al., 1982, 1984; Heyworth et al., 1984), presumably by modifying changes occurring at the proximal effector coupling site. Certainly, here we observed a dramatic effect of pertussis-toxin pre-

Table 2 Action of benzyl alcohol on the activation of adenylate cyclase by p[NH]ppG

p[NH]ppG was used at a concentration of 100 μ M in all of the experiments described below. Unless stated otherwise, all assays were done at 25 °C. Data are expressed as a change relative to the lag time seen with 100 M p[NH]ppG + 5 mM MgCl₂ at 25 °C, whose magnitude was 5.7 $\pm$ 0.6 min (S.D., n = 6 different membrane preparations). *Activities expressed as a percentage of that activity observed in the presence of 100 μ M p[NH]ppG + 5 mM MgCl₂ and assayed at 25 °C. †Experiments done with 100 μ M p[NH]ppG + 5 mM MgCl₂, but assayed at 35 °C. ‡Activities expressed as a percentage of that activity observed in the presence of 100 μ M p[NH]ppG + 5 mM MgCl₂ and assayed at 35 °C. Each experimental set was done three times using different membrane preparations with adenylate cyclase assays in triplicate and with errors given as s.d. Abbreviation: n.e., no evident lag (< 0.5 min).

Ligand	Lag period (min)	Relative lag period (%)	Steady-state rate (pmol/min per mg of protein)	Steady-state rate (%)
+ 50 mM benzyl alcohol	2.5 $\pm$ 0.4	44	3861 $\pm$ 274	394
+ 80 mM benzyl alcohol	1.8 $\pm$ 0.3	32	4988 $\pm$ 382	509
+ 50 mM benzyl alcohol + 100 mM NaCl	4.8 $\pm$ 0.4	84	1391 $\pm$ 89	142
Assayed at 35 °C†	3.8 $\pm$ 0.4	67*	2827 $\pm$ 835	[100]‡
Assayed at 35 °C†	n.e. (< 0.5)	0	6417 $\pm$ 593	[227]‡
+ 50 mM benzyl alcohol Assayed at 35 °C	n.e. (< 0.5)	0	7039 $\pm$ 627	[249]‡
+ 80 mM benzyl alcohol				

treatment in decreasing the lag time (Table 1). As the sole target for pertussis-toxin action in platelets is G_i, then this clearly identifies a pivotal role for this G-protein in influencing the activation of G_s. The absence of a 'pool' of $\beta\gamma$ -subunits arising from the dissociation of G_i might thus allow for the accelerated dissociation of G_s and a decrease in the degree of hysteresis shown by the process.

A fourth approach involved either replacing 5 mM Mg²⁺ with 5 mM Mn²⁺ in the assay, or using high [Mg²⁺] (25 mM) to attenuate the functioning of G_i. These conditions serve to promote G_s dissociation and hence to 'uncouple' or minimize the effect of G_i (Hoffman et al., 1981; Cooper, 1982; Jakobs et al., 1983). Consistent with the studies reported above, we were able to effect a marked decrease in the lag time for activation of adenylate cyclase by p[NH]ppG (Table 1) under conditions where we observed the complete loss (> 965%) of α_2 -adrenergic inhibition of adenylate cyclase activity when stimulated by either prostaglandin E₁ or forskolin.

A fifth strategy employed the local anaesthetic and membrane-fluidizing agent benzyl alcohol, as we have suggested (Spence and Houslay, 1990) that it can selectively alter G_i function by disrupting $\beta\gamma$ -subunit interaction with G_s, hence promoting the dissociation and activation of this G-protein. Consistent with this contention, we show here (Table 2; Figure 1) that benzyl alcohol markedly decreased the lag time. Indeed, 50 mM benzyl alcohol was capable of reversing the effect that 100 mM NaCl exerted in lengthening the lag time (Table 2) in what appeared to be a simple additive fashion. For, when added to assays alone, 100 mM NaCl increased the lag time by about 44% (Table 2), and 50 mM benzyl alcohol decreased the lag by about 56% (Table 2). Thus, assuming a mutual antagonism, one would predict that, when added together, a decrease in the lag time of ~12% would be observed. Such a calculated value compares favourably with the decrease of ~16% found experimentally (Table 2). Note also that increasing the temperature at which assays were done also served to decrease the lag period (Table 2).

Benzyl alcohol also caused a dramatic (around 4-fold) increase in the steady-state rate of p[NH]ppG-stimulated adenylate cyclase activity (Table 2). This could be due either to its ability to increase membrane fluidity, an action established as being able to elicit an increase in adenylate cyclase activity, or, alternatively, a direct stimulatory effect on the protein components (see Houslay and Gordon, 1983). In contrast, 100 mM NaCl had little effect (Table 1) on the steady-state rate of p[NH]ppG-stimulated adenylate cyclase activity (< 20%), despite its pronounced action on the lag time. Nevertheless, 100 mM NaCl attenuated the stimulatory effect of benzyl alcohol on p[NH]ppG-stimulated adenylate cyclase activity (Table 2), suggesting that NaCl antagonized the conformational change elicited by the local anaesthetic.

Our studies have exploited the ability of the non-hydrolysable GTP analogue p[NH]ppG to cause the hysteretic activation of adenylate cyclase in order to identify a pivotal role for G_i in modulating the rate of G_s-mediated activation of adenylate cyclase in a 'native' biological membrane. These studies show that the degree of hysteresis observed can be altered profoundly by changes in G_i function. It is possible that the rate-limiting step for the activation of G_s is its dissociation and that G_i may influence this, through Mass Action, by increasing the size of $\beta\gamma$ -subunit 'pool'. This would be consistent with the pre-treatment of membranes with cholera toxin abolishing the lag, as this toxin causes the dissociation of G_s (Kahn and Gilman, 1984). That such a key process is influenced markedly by the local anaesthetic and membrane-fluidizing agent benzyl alcohol may indicate that certain of the biological effects of local anaesthetics could arise from perturbations of G-protein-controlled processes.

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