The role of GTP in prostaglandin E₁ stimulation of adenylate cyclase in platelet membranes

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Adenylate cyclase activity in platelet membrane preparations was measured in the presence of prostaglandin E₁ (PGE₁), GTP and a non-hydrolysable analogue of GDP, guanosine 5'-[β -thio]diphosphate (GDP[β S]). A dose-dependent inhibition of adenylate cyclase by GDP[β S] was observed that could be reversed either by adding increased amounts of GTP or of PGE₁.

The activation of adenylate cyclase in the plasma membrane of a wide variety of vertebrate cells by many hormones has been shown to be dependent on the presence of GTP (Ross & Gilman, 1980). In platelet plasma membranes adenylate cyclase is activated by PGE₁ (Jakobs *et al.*, 1976). However, the extent to which this activation requires GTP is not entirely clear. For example, the stimulation of platelet adenylate cyclase by PGE₁ has been variously reported to be slightly activated by GTP (Jakobs, 1978), to be strongly dependent on the presence of GTP (Krishna *et al.*, 1972) or to activate in the presence of low concentrations of GTP and to inhibit at high concentrations (Steer & Wood, 1979). Despite these divergent reports it is clear that in every system PGE₁ can substantially activate platelet adenylate cyclase without added GTP. The differences may reflect variations in the source and method of preparation of the platelets and of the procedures used for preparing platelet membranes. An additional reason may be that various amounts of GTP can contaminate the ATP substrate used in the adenylate cyclase assay (Kimura *et al.*, 1976).

We therefore sought an alternative approach that would establish unequivocally the exact role of GTP in the regulation of adenylate cyclase by PGE₁ in platelets. GDP[β S] is an analogue of GDP that cannot be phosphorylated to GTP. In the presence of hormone, GDP[β S] competes with GTP and other guanine nucleotides to inhibit adenylate cyclase (Eckstein *et al.*, 1979). In the present study we have used GDP[β S] to test the extent to which activation by PGE₁ in the absence of added GTP depends upon endogenous GTP.

Materials and methods

Materials

ATP, cyclic AMP, GTP, GDP[β S] and creatine kinase were supplied by Boehringer, Lewes, East Sussex, U.K. Phosphocreatine and PGE₁ were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Cyclic [³H]AMP and [³²P]P_i were obtained from Amersham International, Amersham, Bucks., U.K. [α -³²P]ATP was prepared by the method of Martin & Voorheis (1977).

Methods

Preparation of platelet plasma membranes. Plasma membranes were prepared from platelet concentrates supplied by the East Anglian Regional Transfusion Service, Cambridge, U.K. The platelets had been stored for 4 days at 4°C in acid citrate/dextrose solution (Aster & Jandl, 1964). They were washed by a modification of the procedure of Phillips & Agin (1977) by resuspending in 2 vol. of wash buffer A (NaCl, 120 mM; trisodium citrate, 12.9 mM; glucose, 30 mM), and centrifuged first at 160g for 15 min to remove contaminating red cells and secondly at 1000g for 15 min to pellet the platelets. The pellet was gently suspended in buffer B (NaCl, 154 mM; EDTA, 1 mM; Tris, pH 7.4, 10 mM).

All manipulations were carried out in polypropylene tubes. The platelets were disrupted by freezing in liquid N₂ and thawing twice. The lysate was then homogenized in buffer B, using a Dounce homogenizer with power-driven Teflon pestle at 1650 rev./min. The lysate was centrifuged at 35 000g at 4°C for 30 min, and the pelleted platelet membranes resuspended in fresh buffer B, homogenizing as before. The centrifugation step was carried out a second time, the membranes resuspended in 1 mM-KHCO₃, and stored in liquid N₂.

Abbreviations used: GDP[β S], guanosine 5'-[β -thio]diphosphate; PGE₁, prostaglandin E₁.

Platelet lysates were also prepared from fresh human blood. Here the freshly drawn blood was added to anti-coagulant (citric acid, 1.2%; trisodium citrate, 2.2%) using about 2 ml of anti-coagulant per 10 ml of blood. The blood was centrifuged at 1000 *g* for 3 min and the sedimented red cells were discarded. The platelet-rich plasma was then centrifuged first at 160 *g* and secondly at 1000 *g* as described above in the preparation from platelet concentrates, and the membranes were prepared and stored by the same procedure.

Adenylate cyclase activity was assayed by the method of Salomon *et al.* (1974). Incubations were carried out for 10 min at 37°C, in a volume of 0.1 ml, and contained 15–30 μ g of platelet membrane protein, 25 mM-Tris/HCl buffer, pH 7.4, 0.1 mM-ATP, 1 μ Ci of [α - 32 P]ATP, 0.1 mM-cyclic AMP, 10 mM-MgCl₂, 1 mM-dithiothreitol, 5 mM-phosphocreatine and 5 units of creatine kinase. The activity of adenylate cyclase was linear for at least 10 min under all conditions.

Protein was determined by the method of Lowry *et al.* (1951).

Results and discussion

A comparison between the dose-response relation of adenylate cyclase to PGE₁ in the presence of 0.1 mM-GTP, in membranes prepared from platelet concentrates and from fresh blood is shown in Fig. 1. The responses of membranes obtained from these two sources were similar, with half-maximal stimulation at 0.5 μ M. In the absence of added GTP the stimulation by PGE₁ was less, and half-maximal stimulation was observed at 0.5 μ M-PGE₁. The addition of GDP[β S] further reduced the activation, but half-maximal stimulation was still observed at 0.5 μ M-PGE₁ (results not shown).

Values for cyclic AMP production from platelet membranes stimulated with maximal amounts of PGE₁ (10 μ M) in the presence of GTP were comparable with values reported by Aktories *et al.* (1982) and Stiles & Lefkowitz (1982). Subsequent experiments (Table 1; Figs. 2 and 3) were carried out using membranes from platelet concentrates.

The activation of adenylate cyclase by PGE₁ in the presence and in the absence of GTP is shown in Table 1. GTP increased by the activation of adenylate cyclase by PGE₁. The stimulation obtained with both effectors added together was greater than the sum of the stimulation seen with each effector added alone. However, there was a very marked stimulation of adenylate cyclase by PGE₁ in the absence of added GTP.

The effect of systematically varying the concentration of both GTP and GDP[β S] on the activity of adenylate cyclase in the presence of PGE₁ (1 μ M) is shown in Fig. 2. In the absence of GDP[β S] the

Fig. 1. Dose-response relation of human platelet membrane preparations to prostaglandin PGE₁.

Membranes from platelet concentrates (23 μ g of protein/assay) (O) and from fresh platelets (18 μ g of protein/assay) (●) were incubated for 10 min at 37°C with GTP (0.1 mM) and with PGE₁ as indicated. Adenylate cyclase activity was determined as described in the Materials and methods section. Each point is the mean value from three separate incubations + S.E.M.

addition of GTP gave a relatively small increase in activity at concentrations up to 0.1 μ M. However, at 1 mM-GTP, some reduction in activity was observed. The addition of GDP[β S] caused a marked dose-dependent reduction in the adenylate cyclase activity in both the presence and the absence of added GTP. As the concentration of GDP[β S] was increased higher concentrations of GTP were needed to achieve maximal activation. Basal adenylate cyclase activity was decreased by 50% by 0.1 mM-GDP[β S].

Fig. 3 shows the effect of various concentrations of PGE₁ and GTP in the presence of a constant concentration of GDP[β S] (0.1 mM). As the concentration of PGE₁ was increased the inhibitory effect of GDP[β S] was progressively overcome. The

Table 1. *Effect of GTP and of PGE₁ on adenylate cyclase activity in platelet membranes*

Platelet membranes (18 μ g of protein/assay) were incubated for 10 min at 37°C in the presence and absence of each of GTP and PGE₁ at the concentrations indicated. Values are from three separate incubations and are means $\pm$ S.E.M.

[GTP] (M)	[PGE ₁] (M)	Cyclic AMP (pmol/min per mg)
—	—	27.7 $\pm$ 1.1
—	10 ⁻⁶	162.5 $\pm$ 13.7
10 ⁻⁶	—	36.2 $\pm$ 2.3
10 ⁻⁶	10 ⁻⁶	275.2 $\pm$ 14.8
10 ⁻⁵	—	34.0 $\pm$ 2.9
10 ⁻⁵	10 ⁻⁶	305.2 $\pm$ 10.6

Fig. 2. *Effect of various concentrations of GDP [β S] and GTP on PGE₂-stimulated adenylate cyclase activity* Platelet membranes (20 μ g of protein/assay) were incubated for 10 min at 37°C with PGE₁ (1 μ M) and with GTP at the concentrations indicated. GDP[β S] concentrations were: ○, none; ●, 0.1 mM; □, 0.3 mM; ■, 1 mM. Δ , Basal activity in the absence of any additions. Each point is the mean value from three separate incubations $\pm$ S.E.M.

Fig. 3. *Effect of increasing concentrations of PGE₁ and of GTP on adenylate cyclase activity in platelet membranes incubated with a constant concentration of GDP[β S]* Platelet membranes (20 μ g of protein/assay) were incubated for 10 min at 37°C with GDP[β S] (0.1 mM), and with GTP at the concentrations indicated. PGE₁ concentrations were: ○, 0.1 μ M; ●, 0.5 μ M; □, 1 μ M; ■, 10 μ M. Each point is the mean value from three separate incubations $\pm$ S.E.M.

stimulation by PGE₁ was augmented by increasing concentrations of GTP. This shows that the inhibition observed with GDP[β S] was reversible and specific since at all levels of stimulating hormone the inhibition of GDP[β S] could be overcome by increased amounts of GTP.

It is clear that GDP[β S] inhibits the stimulation of platelet membrane adenylate cyclase in the presence of GTP. As the amount of added GTP was increased (Fig. 2), proportionally greater amounts of

GDP[β S] were required to achieve the same amount of inhibition; this behaviour would be expected for an activator and inhibitor acting through a single regulatory site. In the absence of added GTP the stimulation of adenylate cyclase by PGE₁ was also inhibited by GDP[β S] in a dose-dependent fashion and was virtually abolished at a concentration of 1 mM.

This suggests that stimulation by PGE₁ in the absence of added GTP involves the GTP-binding protein of adenylate cyclase and requires endogenous GTP.

Endogenous GTP could be present in the membrane preparations or in the ATP used in the assay medium. Several groups have also reported the presence of protein factors in a number of tissues that appear to have a similar effect to GTP (Doberska & Martin, 1978; Crawford *et al.*, 1980). The use of GDP[β S] gives a similar test for the involvement of a GTP-binding protein without the need to determine the source of the GTP or protein factor causing the GTP-like effect.

We conclude that the effect of PGE₁ on platelet adenylate cyclase is dependent on the presence of GTP and that the stimulation in the absence of added GTP reflects the presence of GTP in the membrane preparation and in the ATP used as substrate for the enzyme.

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References

- Aktories, K., Schultz, G. & Jakobs, K. H. (1982) *FEBS Lett.* **146**, 65–68
- Aster, R. H. & Jandl, J. H. (1964) *J. Clin. Invest.* **43**, 843–855
- Crawford, A., MacNeil, S., Amirasooli, H. & Tomlinson, S. (1980) *Biochem. J.* **188**, 401–407
- Doberska, C. & Martin, B. R. (1978) *FEBS Lett.* **82**, 273–277
- Eckstein, F., Cassel, D., Levkovitz, H., Lowe, M. & Selinger, Z. (1979) *J. Biol. Chem.* **254**, 9829–9834
- Jakobs, K. H. (1978) *FEBS Symp. S7* **54**, 11–20
- Jakobs, K. H., Saur, W. & Schultz, G. (1976) *J. Cyclic Nucleotide Res.* **2**, 381–392
- Kimura, N., NaKane, K. & Nagata, N. (1976) *Biochem. Biophys. Res. Commun.* **70**, 1250–1256
- Krishna, G., Harwood, J. P., Barber, A. J. & Jamieson, G. A. (1972) *J. Biol. Chem.* **247**, 2253–2254
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Martin, B. R. & Voorheis, H. P. (1977) *Biochem. J.* **161**, 555–559
- Phillips, D. R. & Agin, P. P. (1977) *J. Biol. Chem.* **252**, 2121–2126
- Ross, E. M. & Gilman, A. G. (1980) *Annu. Rev. Biochem.* **49**, 533–564
- Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548
- Steer, M. L. & Wood, A. (1979) *J. Biol. Chem.* **254**, 10791–10797
- Stiles, G. L. & Lefkowitz, R. J. (1982) *J. Biol. Chem.* **257**, 6287–6291