Platelet activating factor and U44069 stimulate a GTPase activity in human platelets which is distinct from the guanine nucleotide regulatory proteins, N_s and N_i

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Platelet-activating factor (PAF, 2-acetyl-1-alkyl-*sn*-glycero-3-phosphocholine) and the stable thromboxane-receptor agonist U44069 (9 α ,11 β -epoxymethanoprostaglandin H_2) stimulated GTPase activity in platelet membranes in a dose-dependent fashion, yielding K_a values of 12 nM and 27 nM respectively. The degree of GTPase activation elicited by these agents was found to be additive with the GTPase activation due to either the stimulatory (N_s) or inhibitory (N_i) guanine nucleotide regulatory proteins when activated by prostaglandin E_1 and adrenaline (+propranolol) respectively. Treatment of membranes with either cholera or pertussis toxins, which inhibited markedly the receptor-mediated stimulation of the GTPase activities of N_s and N_i respectively, had no or only a small effect, respectively, on the GTPase activity stimulated by PAF and U44069. It is suggested that PAF and U44069, which stimulate inositol phospholipid metabolism in platelets, exert actions through a guanine nucleotide regulatory protein which is distinct from N_s and N_i .

INTRODUCTION

Many receptors generate an intracellular response by modulating the activity of adenylate cyclase and hence controlling intracellular cyclic AMP concentrations. These various specific receptors fall into two categories, responsible for either stimulating (R_s) or inhibiting (R_i) adenylate cyclase. Each family of receptors is now known to exert its effect on adenylate cyclase through a distinct species of guanine nucleotide regulatory protein. Thus stimulatory receptors function via N_s (G_s) and inhibitory receptors through N_i (G_i) (see Limbird, 1981; Houslay, 1984; Gilman, 1984; Jakobs *et al.*, 1985). These proteins have been purified to apparent homogeneity and shown to be heterotrimers with distinct α subunits (see Gilman, 1984; Houslay, 1984).

It is now appreciated that both N_s and N_i require GTP, or suitable analogue, in order to function. An essential feature of their mechanism of action is that an agonist-occupied receptor promotes the binding of GTP to the guanine nucleotide regulatory protein, which is subsequently activated (Gilman, 1984; Houslay, 1984; Birnbaumer *et al.*, 1985; Jakobs *et al.*, 1985). The hydrolysis of GTP, which follows thereon, is believed to play an inherent part in the resultant deactivation sequence (Cassel & Selinger, 1977). The identification and evaluation of N_s and N_i functioning has been greatly aided by the use of cholera toxin and pertussis toxin (islet-activating protein), which cause the ribosylation of N_s and N_i respectively. This leads to the activation of N_s by cholera toxin and the inactivation of N_i by pertussis toxin, with the resultant obliteration of receptor-mediated effects on these guanine nucleotide regulatory proteins (Gilman, 1984; Houslay, 1984; Birnbaumer *et al.*, 1985).

Stimulated (poly)phosphatidylinositol metabolism rep-

resents a key signalling mechanism for a wide variety of receptor species (see, e.g., Berridge, 1984; Downes & Michell, 1985). Evidence has suggested that the receptor-mediated promotion of polyphosphoinositide metabolism might involve a novel form of guanine nucleotide regulatory protein, termed N_p (Gomperts, 1983; Haslam & Davidson, 1984; Litosch *et al.*, 1985). We have used human platelets as a model system to gain insight into this possibility, as they exhibit a GTPase activity which can be stimulated by ligands which activate N_s and N_i . Thus PGE $_1$ stimulates N_s -GTPase activity (Lester *et al.*, 1982) and adrenaline stimulates N_i -GTPase activity through α_2 -adrenoceptors (Aktories & Jakobs, 1981). Neither of these receptors, however, stimulates polyphosphatidylinositol metabolism. In the present study we show that two agents which do stimulate polyphosphoinositide metabolism, namely the stable thromboxane (TP)-receptor agonist U44069 (9 α ,11 β -epoxymethanoprostaglandin H_2) and platelet-activating factor (PAF, 2-acetyl-1-alkyl-*sn*-glycero-3-phosphocholine), stimulate a GTPase activity that appears to be distinct from both N_i and N_s .

MATERIALS AND METHODS

U44069, PAF and PGE $_1$ were kindly given by Dr. Euan MacIntyre, Department of Pharmacology, University of Glasgow. Cholera toxin, adrenaline and propranolol were from Sigma Chemical Co., Poole, Dorset, U.K. Purified pertussis toxin was from PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts., U.K. All other biochemicals were from Boehringer (U.K.) Ltd., Lewes, East Sussex, U.K. Radiochemicals were from Amersham International,

Abbreviations used: PGE $_1$, prostaglandin E_1 ; TP, thromboxane; PAF, platelet-activating factor.

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Amersham, Bucks., U.K. All other chemicals were of A.R. grade from BDH Chemicals, Poole, Dorset, U.K.

Blood was obtained from the antecubital vein of healthy volunteers who were not undergoing medication and had not undergone medication for at least 1 month before the experiments. Platelets were prepared as described by MacIntyre & Pollock (1983). Briefly, blood was collected in 0.38% (w/v) trisodium citrate, as an anticoagulant, and centrifuged at 1000 *g* for 5 min at room temperature to obtain, as a supernatant, platelet-rich plasma, which was aspirated into plastic tubes. This supernatant was centrifuged at 30000 *g* for 15 min at 4 °C, and the resulting pellet was resuspended in ice-cold 10 mM-Tris/HCl buffer containing 1 mM-EDTA, final pH 7.4 (lysing buffer). Membranes were prepared from this pellet as in Jakobs *et al.* (1982). Briefly, the pellet was homogenized in this buffer with ten up-and-down strokes of a motor-driven Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 30000 *g* for 15 min at 4 °C and the resultant pellet washed by centrifugation a further time after resuspension in the lysing buffer. The final pellet was resuspended in 10 mM-Tris/HCl buffer, pH 7.4, and frozen and stored in batches at -80 °C.

GTPase activity was measured by following the liberation of [³²P]P_i from [α-³²P]GTP, essentially as described by Cassel & Selinger (1976). The assay, in a final volume of 100 μl, contained 0.4 μM-[γ-³²P]GTP, 5 mM-MgCl₂, 100 mM-NaCl, 1 mM-dithiothreitol, 0.5 mM-EGTA, 1 mM-ATP, 50 units of creatine kinase/ml, 12 mM-phosphocreatine and 50 mM-Tris/HCl buffer, pH 7.4, as well as the appropriate drugs. The reaction was initiated by the addition of membranes and carried out for 10 min at 30 °C, over which time linear time courses were obtained under all conditions and initial rates assessed. The reaction was terminated by addition of 0.5 ml of 20 mM-sodium phosphate buffer,

pH 7.4, containing 5% (w/v) Norit-A charcoal. The tubes were vortex-mixed and centrifuged at 12000 *g* for 5 min, and 0.2 ml samples of the supernatant were taken for scintillation counting. Reactions were performed in triplicate together with controls with no added membranes.

Platelet membranes were treated with either cholera or pertussis toxin that had been thiol-preincubated by using a modification of the method of Hildebrandt *et al.* (1983), as described by us previously in some detail (Heyworth *et al.*, 1985). Briefly, platelet membranes were incubated in 'ribosylation' mixture containing 1 mM-NAD⁺, 0.5 mM-GTP, 15 mM-thymidine and 100 mM-potassium phosphate buffer, final pH 7.5. For treatment with cholera toxin the incubation was performed for 10 min at 30 °C with 100 μg of cholera toxin/ml, and with pertussis toxin for 30 min at 37 °C with 50 μg of toxin/ml. In each case appropriate control incubations were done in the absence of toxin. After the incubation the membranes were pelleted by centrifugation at 12000 *g* for 15 min at 4 °C and washed twice by centrifugation with resuspension in the GTPase assay mixture. Membranes were kept on ice before use.

RESULTS AND DISCUSSION

As has been shown by others (Lester *et al.*, 1982; Aktories *et al.*, 1982), PGE₁ stimulated platelet GTPase activity (Table 1). This was clearly a function of N_s, as such a stimulated activity could be blocked by treatment of membranes with cholera toxin (Table 2). Platelets exhibit α-adrenoceptors, which act to inhibit adenylate cyclase through N_i, as well as a small fraction of β-adrenoceptors (Jakobs *et al.*, 1985). In order to block β-adrenoceptor functioning, we added propranolol to all experiments involving adrenaline. Here we see that

Table 1. Ligand-stimulated GTPase activities in human platelets

All ligands were used at saturating concentrations which elicited maximal GTPase stimulation. These were PGE₁ (100 μM), PAF (1.8 μM), U44069 (1 μM) and adrenaline (100 μM). In order to block any possible β-adrenoceptor action, propranolol (1 mM) was added with adrenaline. Results are expressed as percentage stimulation over basal GTPase activity, which was 43.8 ± 2.8 pmol/min per mg of protein. The additivity ratio was calculated by expressing the observed (%) stimulation of GTPase activity seen with mixtures of ligands as a ratio of the stimulation expected for full additivity, i.e. separate systems. The additivity ratio will thus be equal to 1.00 for fully independent stimulating ligands and 0.5 for ligands which are each able to activate fully a common system. Values in parentheses signify the additivity ratio calculated by using the percentage stimulation observed with a mixture of PAF + U44069 together in the assay rather than summing the values for stimulation observed when these ligands were added independently; *significantly lower than 1.00 at *P* < 0.001. Data are means ± s.d. for four separate experiments (*n* = 4), with triplicate determinations of GTPase activity.

Ligand	Stimulation over basal activity (%)	Additivity ratio
PGE ₁	46 ± 4	—
Adrenaline + propranolol	31 ± 3	—
PAF	30 ± 1	—
U44069	34 ± 3	—
PGE ₁ + (adrenaline + propranolol)	75 ± 2	0.97
PGE ₁ + PAF	82 ± 5	1.08
PGE ₁ + U44069	82 ± 4	1.03
PGE ₁ + PAF + U44069	89 ± 5	0.81
PGE ₁ + (PAF + U44069)	(89 ± 5)	(0.93)
(Adrenaline + propranolol) + PAF	59 ± 3	0.97
(Adrenaline + propranolol) + U44069	64 ± 2	0.98
(Adrenaline + propranolol) + PAF + U44069	81 ± 6	0.85
(Adrenaline + propranolol) + (PAF + U44069)	(81 ± 6)	(1.00)
PAF + U44069	50 ± 2	(0.78)*

Table 2. Action of cholera and pertussis toxins on ligand-stimulated GTPase activities in human platelets

Results are means $\pm$ S.D. for three separate experiments ($n = 3$), with triplicate determinations of GTPase activity. Isolated platelet membranes were treated with a 'ribosylation' mixture in the presence and absence of thiol-preactivated toxins as in the Materials and methods section.

Ligand	Change in GTPase stimulation (%)	
	In pertussis-toxin-treated membranes	In cholera-toxin-treated membranes
PGE ₁	0 $\pm$ 3	-90 $\pm$ 6
Adrenaline + propranolol	-67 $\pm$ 5	1 $\pm$ 2
PAF	-18 $\pm$ 3	-9 $\pm$ 5
U44069	-14 $\pm$ 2	-1 $\pm$ 3

adrenaline stimulates N₁-GTPase activity, and this effect was markedly decreased by pre-treating membranes with pertussis toxin (Table 2). Indeed, complete abolition of this activity could be effected by using a longer incubation time, although, under such circumstances, the basal GTPase activity was also somewhat decreased (results not shown). Thus experiments were performed under the conditions described where control basal GTPase activity remained unaltered during the time of the experiment (approx. 5%). Consistent with PGE₁ and adrenaline exerting actions on distinct G-proteins, we see that treatment of platelet membranes with saturating concentrations of these ligands led to simple additivity in the observed stimulated GTPase activity.

U44069 and PAF stimulated GTPase activity in human platelets in a dose-dependent fashion (Fig. 1), with K_a values of 27 ± 7 nM and 12 ± 3 nM respectively (S.D., $n = 3$). These values are very similar to those observed for the stimulation of polyphosphoinositide metabolism by these ligands (54 nM and 35 nM respectively) in intact platelets (MacIntyre & Pollock, 1983; Pollock *et al.*, 1984).

When saturating concentrations of either of these ligands were used to stimulate GTPase activity in the presence of either PGE₁ or adrenaline, simple additive results were obtained (Table 1). If both PAF and U44069 were added together, however, a less than additive stimulatory effect on GTPase activity was exerted (Table 1).

We also evaluated mixtures of PAF + U44069 + adrenaline and PAF + U44069 + PGE₁. If the additivity ratio for the mixtures was calculated as a ratio of GTPase stimulation elicited when each of these stimulants was added in isolation to GTPase assays, then a value of much less than 1 was observed (Table 1), indicating a lack of additivity. However, as discussed above, a mixture of PAF + U44069 yielded a less than additive stimulation of GTPase activity, implying an action on a common G-protein. If the observed GTPase activity for the PAF + U44069 mixture, i.e. the activity that might not be due to either N_s or N₁, was used to calculate the additivity ratio for mixtures of PAF + U44069 with either adrenaline or PGE₁, a clearly additive result was then apparent (Table 1). These observations imply that PAF and U44069 might be capable of stimulating a distinct guanine nucleotide regulatory protein (G-protein) that is distinct from N_s and N₁, stimulated by PGE₁ and adrenaline (α_2 -

adrenoceptor) respectively. However, it is possible that these ligands could stimulate either N_s or N₁, but that no fully occupied receptor population can activate all that particular G-protein pool. As PAF (Haslam & Vanderwel, 1982) and U44069 (M. D. Houslay & A. Wilson, unpublished work) inhibit rather than stimulate adenylate cyclase activity, it is extremely unlikely that these ligands

Fig. 1. U44049 and PAF stimulate a GTPase activity in human platelets

GTPase assays were performed at 30 °C for 5 min as described in the Materials and methods section. Data shown are means $\pm$ S.D. for three separate experiments ($n = 3$), with triplicate assays of GTPase activity.

can activate N_s . As N_i activation by α -adrenoceptors causes inhibition of adenylate cyclase through N_i (Aktories *et al.*, 1982), but such receptors do not stimulate polyphosphoinositide metabolism, it is also unlikely that U44069 and PAF exert their action through N_i . The possibility that PAF and U44069 stimulate a distinct guanine nucleotide regulatory protein receives strength from our observations (Table 2) that, firstly, cholera-toxin treatment had no effect on the ability of U44069 and PAF to stimulate GTPase activity, whereas it obliterated that due to PGE_1 functioning through N_s . Secondly, under conditions where pertussis toxin effected a near-70% block on the increased GTPase activity elicited by α_2 -adrenoceptor stimulation of N_i , then GTPase stimulation by PAF and U44069 was inhibited by only 14–18%.

Our results show that the receptors for both PAF and the TP receptor can stimulate GTPase activity in human platelets. We suggest that these receptors interact with a common guanine nucleotide regulatory protein which is distinct from both N_i and N_s . These experiments provide further support for the contention (Gomperts, 1983) that a distinct guanine nucleotide regulatory protein may be involved in mediating receptor-stimulated polyphosphoinositide metabolism.

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