Thrombin, unlike vasopressin, appears to stimulate two distinct guanine nucleotide regulatory proteins in human platelets

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The thrombin-stimulated GTPase activity of human platelets was additive with respect to the GTPase stimulation effected by prostaglandin E_1 , but not with that stimulated by adrenaline, vasopressin and platelet-activating factor (PAF). Treatment of platelet membranes with pertussis toxin partially inhibited the thrombin-stimulated GTPase, but had no effect on the vasopressin-stimulated GTPase activity, whereas cholera toxin treatment had no effect on either of these stimulated GTPase activities. Thrombin, adrenaline and PAF, but not vasopressin, inhibited the adenylate cyclase activity of isolated plasma membranes through the action of N_i only, this being inhibited by pertussis toxin. It is suggested that thrombin exerts effects through both the inhibitory guanine nucleotide regulatory protein N_i and through the putative guanine nucleotide regulatory protein, N_p , involved in regulating receptor-stimulated inositol phospholipid metabolism. However, vasopressin appears to exert its effects solely through the putative N_p .

INTRODUCTION

Platelets contain receptors for ligands capable of both stimulating and inhibiting adenylate cyclase (Jakobs *et al.*, 1985). These actions are mediated by distinct guanine nucleotide regulatory proteins. Thus N_s and N_i mediate the action of stimulatory and of inhibitory hormones respectively on adenylate cyclase (Gilman, 1984; Houslay, 1984). The ability of agonists to stimulate the GTPase activity of these regulatory proteins can be blocked by the NAD-dependent ADP-ribosylation of N_s and N_i with cholera toxin and pertussis toxin respectively (Houslay, 1984). More recently, considerable evidence has been presented which suggests that a distinct guanine nucleotide regulatory protein, N_p , links specific receptors to the stimulation of inositol lipid metabolism (Gomperts, 1983; Haslam & Davidson, 1984; Cockcroft & Gomperts, 1985; Litosch *et al.*, 1985). Indeed, in platelets, PAF (platelet-activating factor; 2-acetyl-1-alkyl-*sn*-glycero-3-phosphocholine) and U44069 ($9\alpha,11\beta$ -epoxymethanoprogesterone) both activate polyphosphatidylinositol metabolism (MacIntyre & Pollock, 1983; Pollock *et al.*, 1984) and appear to stimulate predominantly a GTPase activity which is distinct from both N_s and N_i and has been suggested to reflect the activity of the putative N_p (Houslay *et al.*, 1986).

It has been the contention of Jakobs and co-workers (Aktories & Jakobs, 1984) that thrombin acts on platelets solely through N_i activation. However, as well as inhibiting adenylate cyclase (Aktories & Jakobs, 1984), thrombin also stimulates polyphosphatidylinositol metabolism (Bell & Majerus, 1980; Billah & Lapetina, 1982; Agranoff *et al.*, 1983). We show here that thrombin appears to interact with both N_i and N_p in platelets.

MATERIALS AND METHODS

Thrombin, PAF and PGE_1 were kindly given by Dr. Euan MacIntyre, Department of Pharmacology, Univer-

sity of Glasgow. Cholera toxin, adrenaline and propranolol were from Sigma Chemical Co., Poole, Dorset, U.K. Purified pertussis toxin was either given by Dr. J. Freer, Department of Microbiology, University of Glasgow, or from PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts., U.K. Vasopressin was obtained from BRL, Paisley, Scotland, U.K. All other biochemicals were from Boehringer (U.K.) Ltd., Lewes, East Sussex, U.K. Radiochemicals were from Amersham International, 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 normal 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) and membranes were prepared as described by Jakobs *et al.* (1982).

GTPase activity was measured as described by Houslay *et al.* (1986), by monitoring the liberation of [^{32}P]P_i from [γ - ^{32}P]GTP, essentially as described by Cassel & Selinger (1976). The assay, in a final volume of 100 μ l, contained 0.4 μ M- $[\gamma$ - ^{32}P]GTP, 5 mM-MgCl₂, 100 mM-NaCl, 1 mM-dithiothreitol, 0.5 mM-EGTA, 1 mM-ATP, 50 mM-Tris/HCl buffer, pH 7.4, and the appropriate drugs. As described by Cassel & Selinger (1976), the high-affinity component was obtained by carrying out the assays in the presence and absence of added unlabelled GTP (100 μ M). Linear time courses, at 30 °C, were obtained in all instances over a 10 min period, during which initial rates were assessed.

Adenylate cyclase assays were carried out as described by Houslay *et al.* (1976), except that 100 mM-NaCl was added to the reaction mixtures with final pH 7.4, containing also 1.5 mM-ATP, 5 mM-MgSO₄, 10 mM-theophylline, 7.4 mg of phosphocreatine/ml, 1 mg of creatine kinase/ml and 25 mM-triethanolamine buffer, final pH 7.4. The cyclic AMP produced was assessed as described previously (Whetton *et al.*, 1983), by using the

Abbreviations used: PAF, platelet-activating factor; PG, prostaglandin.

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Table 1. Thrombin stimulates GTPase activity in human platelets

Saturating concentrations of each ligand were used such that no further augmentation of GTPase activity was elicited by any increase in concentration. The concentrations employed were adrenaline (100 μ M), PAF (1.8 μ M), PGE₁ (100 μ M), thrombin (0.25 unit/ml) and vasopressin (10 nM). When adrenaline was used, propranolol (1 μ M) was present to negate any possible β -adrenergic effect. Stimulation is given as the percentage increase in activity over basal GTPase at 30 °C, which was 47 ± 4 pmol/min per mg of protein. The additivity ratio is given as the observed percentage increase seen with mixtures of both ligands present as a fraction of the sum of the stimulatory effects seen when each ligand was present alone. Fully independent systems will yield a ratio of 1.0, and those sharing a common system will yield a value of 0.5. Results are means $\pm$ s.d. with $n = 5$ for separate experiments utilizing triplicate determinations of GTPase activity.

Ligand	Stimulation over basal (%)	Additivity ratio
Thrombin	57 ± 2	
PGE ₁	33 ± 3	
Adrenaline + propranolol	25 ± 3	
PAF	24 ± 2	
Vasopressin	23 ± 2	
Thrombin + PGE ₁	86 ± 3	0.96
Thrombin + adrenaline + propranolol	65 ± 3	0.79
Thrombin + PAF	62 ± 4	0.77
Vasopressin + adrenaline + propranolol	51 ± 4	1.07
Vasopressin + PGE ₁	59 ± 6	1.06
Vasopressin + PAF	26 ± 2	0.55
Vasopressin + thrombin	59 ± 6	0.74

cyclic-AMP-binding subunit of protein kinase prepared from bovine heart in a binding assay.

Platelet membranes were treated with either cholera toxin 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 μ g of the toxin/ml, and with pertussis toxin for 30 min at 37 °C with 50 μ g 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 their use.

RESULTS AND DISCUSSION

PGE₁, as shown by others previously (Lester *et al.*, 1982; Aktories *et al.*, 1982), stimulated the GTPase activity of N_s, the guanine nucleotide regulatory protein which is responsible for activating adenylate cyclase (Table 1). In the experiments shown, treatment of platelet membranes with cholera toxin activated N_s and prevented any further (PGE₁) receptor-mediated GTPase stimulation (Table 2). Adrenaline, in platelets, acts through α_2 -adrenoceptors to inhibit adenylate cyclase (Jakobs *et al.*, 1985) through the activation of N_i, the GTPase activity of this regulatory protein being enhanced by adrenaline (Table 1). The ribosylation of N_i by pertussis toxin (islet-activating protein) negates both the inhibitory effect this hormone exerts on adenylate cyclase and the adrenaline-stimulated increase in GTPase activity of N_i (Houslay *et al.*, 1986; Table 2).

PAF stimulates polyphosphatidylinositol lipid meta-

bolism in human platelets (Pollock *et al.*, 1984). We have provided evidence (Houslay *et al.*, 1986) that PAF stimulates a GTPase activity in platelets which is distinct from both N_s and N_i. This conclusion was drawn from observations that the PAF-stimulated GTPase activity was unaffected by cholera toxin treatment, and only marginally decreased by pertussis toxin treatment, and that PAF elicited an apparently additive increase in GTPase activity when added to platelets together with either PGE₁ or adrenaline. On this basis we suggested (Houslay *et al.*, 1986) that PAF can stimulate the GTPase activity of the putative guanine nucleotide regulatory protein N_p, which is believed (Gomperts, 1983) to mediate the action of receptors which stimulate inositol phospholipid metabolism. We show here (Table 1), for the first time in platelets, that vasopressin, a peptide which, like PAF, stimulates polyphosphatidylinositol lipid metabolism (Siess *et al.*, 1986; Pollock & MacIntyre, 1986), also stimulates a GTPase activity. This GTPase stimulation is presumably due to N_p activation. Indeed, Fain *et al.* (1985) have also demonstrated vasopressin-stimulated GTPase in hepatocytes, where vasopressin stimulates inositol lipid metabolism only (Michell *et al.*, 1979). The GTPase activity stimulated by vasopressin was additive as regards both the PGE₁- and the adrenaline-stimulated GTPases, corresponding to N_s and N_i respectively, but not with PAF (Table 1), which we have suggested stimulates predominantly the putative N_p (Houslay *et al.*, 1986). Indeed, the vasopressin-stimulated GTPase activity was completely unaffected by pre-treatment of platelet membranes with either pertussis toxin or cholera toxin under conditions where the functioning of both N_i and N_s, respectively, was either markedly decreased or even abolished (Table 2).

Thrombin causes both polyphosphatidylinositol lipid turnover (see Agranoff *et al.*, 1983) and adenylate cyclase inhibition (Aktories & Jakobs, 1984) in platelets. Aktories & Jakobs (1984) have suggested that this proteolytic enzyme exerts its actions on platelets by stimulating

Table 2. Cholera toxin and pertussis toxin exert differential effects on ligand-stimulated GTPase activities

Isolated platelet membranes were treated with the appropriate toxin as described in the Materials and methods section. The relative change in function was assessed relative to control membranes. The data are from a representative experiment of one carried out three times. The concentration of forskolin in the assay was 10 μ M, and other ligands were at concentrations given in the legend to Table 1. Results are means $\pm$ S.D. for $n = 3$ separate experiments. Abbreviation: N.A., not applicable.

Effector ligand	Change in GTPase stimulation (%) in membranes treated with:		Change in inhibition (%) of adenylate cyclase caused by pertussis toxin (forskolin-stimulated activity)
	Cholera toxin	Pertussis toxin	
PGE ₁	-59 $\pm$ 7	-3 $\pm$ 2	—
Adrenaline + propranolol	-3 $\pm$ 4	-58 $\pm$ 6	-55 $\pm$ 5
PAF	-4 $\pm$ 3	-12 $\pm$ 2	-69 $\pm$ 8
Thrombin	+3 $\pm$ 3	-26 $\pm$ 2	-50 $\pm$ 14
Vasopressin	+3 $\pm$ 2	+9 $\pm$ 6	N.A.

Table 3. Receptor-mediated inhibition of adenylate cyclase in platelet membranes

Ligands were used at the concentrations specified in the legend to Fig. 1, i.e. saturating, with maximal inhibitory effects being noted. Forskolin was present at 10 μ M and PGE₁ at 100 μ M. Basal adenylate cyclase activity was 210 $\pm$ 9 pmol of cyclic AMP produced/min per mg of protein, with forskolin effecting a 17 $\pm$ 5-fold stimulation and PGE₁ a 21 $\pm$ 5-fold stimulation. The additivity ratio is calculated as a ratio of the percentage inhibitory effect seen with a combination of both inhibitory ligands present against the sum of the percentage inhibitory effects seen with both ligands added separately. Fully independent systems will give a ratio of 1.0, and fully interacting ones a ratio of 0.5. Results are means $\pm$ S.D. for $n = 5$ separate experiments, with triplicate determinations of adenylate cyclase activity. Abbreviation: N.D., not detectable (less than 5% effect).

Ligand	Inhibition (%)		Additivity ratio	
	PGE ₁ -stimulated activity	Forskolin-stimulated activity	(PGE ₁)	(forskolin)
Adrenaline + propranolol	24.3 $\pm$ 4.7	41.3 $\pm$ 9.5	—	—
PAF	18.5 $\pm$ 6.4	36.0 $\pm$ 2.8	—	—
Thrombin	24.5 $\pm$ 8.8	50.1 $\pm$ 7.2	—	—
Vasopressin	N.D.	N.D.	—	—
Thrombin + adrenaline + propranolol	27.3 $\pm$ 5.2	68.6 $\pm$ 1.5	0.56	0.75
Thrombin + PAF	24.9 $\pm$ 4.8	68.8 $\pm$ 2.3	0.58	0.80
Adrenaline + propranolol + PAF	26.1 $\pm$ 6.4	64.2 $\pm$ 3.6	0.61	0.83

N₁; this was based on their observation that pertussis toxin treatment prevented thrombin inhibiting adenylate cyclase. If thrombin only interacted with N₁, then we might expect that the thrombin-stimulated GTPase activity, at the maximally effective dose used here, would not be additive with that caused by adrenaline. This, however, is clearly not so (Table 1), for in such experiments the additivity ratio was well above the value of 0.5 which would be expected for ligands functioning through the same system. This suggests that either adrenaline or thrombin alone is incapable of stimulating the entire N₁ pool, or that thrombin interacts with, and stimulates, another guanine nucleotide regulatory protein. This thrombin-stimulated GTPase cannot be due to N_s, as the thrombin-stimulated GTPase activity both showed simple additivity with the PGE₁-stimulated GTPase response (Table 1) and was also unaffected by cholera toxin (Table 2). However, the thrombin-stimulated GTPase activity was only partially additive with the activity stimulated by either PAF or vasopressin (Table 1), agents which themselves yielded an apparently additive GTPase stimulation with adrenaline (Houslay *et al.*, 1986; Table 1) and are suggested to stimulate N_p. We

therefore propose that thrombin might stimulate the GTPase activity of both N₁ and the putative N_p. This contention is reinforced by studies with pertussis toxin, which only decreased the thrombin-stimulated GTPase by about half of the extent by which it decreased the adrenaline-stimulated (N₁) GTPase. The pertussis-insensitive component presumably reflects the putative N_p, which does not appear to be a good substrate for this toxin, as can be inferred from the lack of any effect on vasopressin-stimulated GTPase in the same experiment (Table 2).

Inhibition of adenylate cyclase is believed to occur through two types of mechanisms. One involves the release of β subunits from N₁, which then inhibit N_s activation, and the other involves a direct effect of the α subunit of N₁ on adenylate cyclase (see Houslay, 1984). Inhibition of adenylate cyclase was therefore assessed by investigating the effect on either the ligand-stimulated activity of adenylate cyclase or on an 'amplified' basal activity by using forskolin. Thus PGE₁ (receptor)-stimulated activity might be expected to be inhibited by both routes, whereas forskolin, a diterpene which activates the catalytic unit directly (Limbird, 1981;

Seamon & Daly, 1981), might only reflect the latter mechanism. Thrombin (Aktories & Jakobs, 1984), adrenaline (Jakobs *et al.*, 1985) and PAF (Haslam & Vanderwel, 1982) have all been shown by others to inhibit human platelet adenylate cyclase. We can confirm these observations here (Table 3) whether we assess inhibition by using either PGE₁ or forskolin stimulation of adenylate cyclase activity. Combinations of maximally effective doses of thrombin, adrenaline and PAF showed, however, little or no apparent additivity as regards inhibition of PGE₁-stimulated activity, indicating that they were able to utilize, in full, a common inhibitory mechanism. Indeed all three of these ligands also exhibited less than additive inhibitory effects on forskolin-stimulated adenylate cyclase activity. This again implies that they employed a common inhibitory mechanism. As discussed above, inhibition of forskolin-stimulated activity may well proceed by a somewhat different mechanism from inhibition of PGE₁-stimulated activity, perhaps involving only a direct inhibitory effect of the α subunit of N_i on the catalytic unit of adenylate cyclase. The differences in the degree of inhibition observed when monitoring adenylate cyclase activity in these two distinct stimulatory states (Table 3) would be in accord with this. Certainly, with forskolin-stimulated activity it would appear that no one of these inhibitory ligands could achieve the maximal inhibitory effect, which was attained only when combinations of these ligands were added. Thus partially additive effects were noted with combinations of these inhibitory ligands (Table 3). Unlike the dissimilar degrees of blockade that pertussis toxin exerted on the GTPase activity stimulated by these three ligands, this toxin blocked their inhibitory effects on adenylate cyclase to a similar extent (Table 2). This implies that each of these three ligands inhibited adenylate cyclase solely through N_i, which is consistent with our observation that vasopressin, suggested to function solely through N_p, did not inhibit adenylate cyclase activity (Table 3).

Our inability to observe inhibition of adenylate cyclase by vasopressin in platelets (Table 3), by using either of the ligand-stimulated activities, contrasts with the observations of Vanderwel *et al.* (1983). However, platelets possess V₁-receptors, which in platelets (Siess *et al.*, 1986), like many other tissues (see Michell *et al.*, 1979; Jard, 1983), mediate stimulation of inositol phospholipid metabolism. Indeed, in a variety of tissues vasopressin binding to V₁-receptors has been quite clearly demonstrated not to inhibit adenylate cyclase activity (Jard *et al.*, 1981; Jard, 1983). The only other type of receptors for vasopressin are V₂-receptors, which actually stimulate adenylate cyclase activity (Sawyer *et al.*, 1983; Jard, 1983). It may be that the particular unusual conditions employed for the adenylate cyclase assays by Vanderwel *et al.* (1983), a GTP-inhibited basal state, led to an aberrant effect. Support for such a contention is gained from their failure to observe any ability of vasopressin to decrease intracellular cyclic AMP concentrations in intact platelets. This contrasts with the ability of adrenaline to lower cyclic AMP concentrations in intact platelets (Lenox *et al.*, 1985). It is also possible that, in that study, vasopressin could have stimulated an inositol phospholipid response which, in the presence of residual C-kinase activity, could have led to the indirect inhibition of adenylate cyclase activity (see Heyworth *et al.*, 1984).

We propose that the simplest interpretation of our data is as follows. Vasopressin is suggested to act solely through the putative N_p, a guanine nucleotide regulatory protein which does not appear to provide a good substrate for either pertussis toxin or cholera toxin and which it is suggested does not inhibit adenylate cyclase. Adrenaline (+propranolol), functioning through α_2 -adrenoceptors, appears to act solely through N_i and does not elicit any polyphosphatidylinositol lipid response, but can inhibit adenylate cyclase. Our present results indicate that the ability of PAF to inhibit adenylate cyclase is mediated via an interaction with N_i, the only activity which was here inhibited by pertussis toxin. However, unlike thrombin, the N_i-stimulated component stimulated by PAF appears to form only a relatively small fraction of the total GTPase activity stimulated by PAF. This is identified by the small decrease in the PAF-stimulated GTPase elicited by pertussis toxin treatment (Houslay *et al.*, 1986; Table 2). The fraction of the total PAF-stimulated GTPase activity which is due to N_i would appear then to form less than 20% of the total, the major fraction being the putative N_p. If the N_i component forms only a small fraction of the total GTPase activity stimulated by PAF, it would account for the essentially additive GTPase response found in mixed-substrate experiments with PAF and adrenaline (Houslay *et al.*, 1986). In contrast with the contention by Aktories & Jakobs (1984), we suggest that thrombin acts to stimulate both N_i and N_p, on the basis of both the additivity experiments and the degree of inhibition of the thrombin-stimulated GTPase elicited by pertussis toxin.

Platelets thus appear to have three distinct guanine nucleotide regulatory proteins which are capable of being regulated by specific receptor species. In instances where one ligand is suggested to interact with two distinct guanine nucleotide regulatory proteins, e.g. thrombin, it will be of importance to ascertain whether this can be achieved by a single receptor type or whether there are multiple receptor species present which couple specifically to one type of guanine nucleotide regulatory protein (see Michell & Houslay, 1986). Indeed, evidence has been presented which demonstrates that two distinct receptors for thrombin may occur in platelets (McGowan & Detwiler, 1986).

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