Evidence for two GTPases activated by thrombin in membranes of human platelets

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Thrombin inhibits adenylate cyclase and stimulates GTP hydrolysis by high-affinity GTPase(s) in membranes of human platelets at almost identical concentrations. Both of these thrombin actions are similar to those observed with agonist-activated α_2 -adrenoceptors coupling to the inhibitory guanine nucleotide-binding protein N_i . However, stimulation of GTP hydrolysis caused by adrenaline (α_2 -adrenoceptor agonist) and by thrombin at maximally effective concentrations was partially additive, whereas with regard to adenylate cyclase inhibition no additive response was observed. Furthermore, treatment of platelet membranes with pertussis toxin, which inactivates N_i and largely abolishes thrombin- and adrenaline-induced adenylate cyclase inhibition and adrenaline-induced GTPase stimulation, decreased the thrombin-induced stimulation of GTP hydrolysis by only about 30%. Additionally, the thiol reagent *N*-ethylmaleimide (NEM) at rather low concentrations abolished thrombin- and adrenaline-induced adenylate cyclase inhibition and adrenaline-induced GTPase stimulation. In contrast, the thrombin-induced stimulation of GTP hydrolysis was decreased by only 30–40% by treatment of platelet membranes with even high concentrations of NEM. Treatment with cholera toxin, which inhibits GTPase activity of the N_s (stimulatory guanine nucleotide-binding) protein, has no effect on thrombin-stimulated GTP hydrolysis. The data suggest that thrombin interaction with its receptor sites in platelet membranes leads to stimulation of two GTP-hydrolysing enzymes. One of these enzymes is apparently N_i and is also activated by agonist-activated α_2 -adrenoceptors and is inactivated by pertussis toxin and NEM treatment. The other GTP-hydrolysing enzyme activated by thrombin may represent a guanine nucleotide-binding protein apparently involved in the coupling of thrombin receptors to the phosphoinositide phosphodiesterase.

INTRODUCTION

We previously described that thrombin is a potent and efficient inhibitor of the adenylate cyclase in human platelet membranes (Aktories & Jakobs, 1984). This thrombin action is apparently mediated by the inhibitory guanine nucleotide-binding protein N_i , since the inhibition is GTP-dependent and since it can be inhibited by treatment of platelet membranes with pertussis toxin and *N*-ethylmaleimide (NEM), similar to the action of adrenaline causing adenylate cyclase inhibition via α_2 -adrenoceptors (Jakobs *et al.*, 1982; Aktories *et al.*, 1983). Furthermore, similar to adrenaline, thrombin-induced inhibition of adenylate cyclase was accompanied by an increased GTP hydrolysis by a high-affinity GTPase(s). Both reactions, inhibition of adenylate cyclase and stimulation of GTP hydrolysis, were observed at almost identical concentrations of thrombin (Aktories & Jakobs, 1984), which suggested that, similar to adrenaline's action (Aktories *et al.*, 1983), the thrombin stimulation of high-affinity GTPase is an action at N_i .

On the other hand, thrombin is one of the most effective activators of the phosphoinositide breakdown in human platelets, whereas adrenaline is very weak or not active at all in this regard (Agranoff *et al.*, 1983; Siess *et al.*, 1984; Rittenhouse & Sasson, 1985). Functional

studies performed in permeabilized platelets suggested that a guanine nucleotide-binding regulatory protein may also be involved in the thrombin stimulation of phosphoinositide metabolism (Haslam & Davidson, 1984*a,b*). Therefore, we re-evaluated the thrombin-induced stimulation of GTP hydrolysis in human platelet membranes, to try to resolve a guanine nucleotide-binding protein other than N_i being activated by thrombin. We report here that thrombin activation of GTP hydrolysis by high-affinity GTPase(s) in human platelet membranes can be discriminated from those induced by adrenaline and prostaglandin E_1 (PGE_1), acting via N_i and N_s respectively, on the platelet adenylate cyclase.

MATERIALS AND METHODS

Materials

The agents used were obtained from sources previously described (Jakobs *et al.*, 1982; Aktories *et al.*, 1983; Aktories & Jakobs, 1984).

Preparation and treatment of platelet membranes

Membranes of human platelets were prepared as previously described, with 5 mM-EDTA present throughout the membrane preparation procedure (Jakobs *et al.*, 1982). Treatment of platelet membranes with pertussis

Abbreviations used: N_i and N_s , the guanine nucleotide regulatory protein that couples inhibitory and stimulatory hormone receptors, respectively, to adenylate cyclase; NEM, *N*-ethylmaleimide; PGE_1 , prostaglandin E_1 .

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toxin was performed as described by Aktories & Jakobs (1984) at a concentration of 50 μg of toxin/ml for 30 min at 37 °C. Cholera-toxin treatment was carried out as described by Aktories *et al.* (1983), at a concentration of 100 μg of toxin/ml for 10 min at 30 °C.

GTPase assay

The reaction mixture contained, if not otherwise indicated, 0.5 μM -[γ - ^{32}P]GTP (0.1 μCi /tube), 2 mM- MgCl_2 , 0.1 mM-EGTA, 0.1 mM-ATP, 5 mM-phosphocreatine, 0.4 mg of creatine kinase/ml, 2 mg of bovine serum albumin/ml and the additions indicated in 50 mM-triethanolamine/HCl, pH 7.4, in a total volume of 100 μl . When adenylate cyclase and GTPase activities were determined simultaneously (see Figs. 1–3), the reaction mixture additionally contained 20 μM -forskolin, 1 mM-3-isobutyl-1-methylxanthine and 0.1 mM-cyclic AMP. The reaction was started by the addition of platelet membranes (8–15 μg of protein/tube) and conducted for 10 min at 25 °C. The reaction was stopped and the released P_i was isolated as described by Aktories *et al.* (1983). When the effect of NEM on GTP hydrolysis was studied, the membranes were preincubated with the indicated concentrations of NEM for 15 min at 25 °C in the presence of the complete reaction mixture except for GTP and hormones. This reaction was stopped by the addition of dithiothreitol at a concentration at least 3-fold higher than the highest NEM concentration used. Thereafter, measurement of GTP hydrolysis was started by the addition of GTP without and with the hormonal factors studied and continued for 10 min at 25 °C. Low-affinity GTPase activity was determined and subtracted from the total GTPase activity by measuring the GTP hydrolysis at 50 μM -GTP as described by Cassel & Selinger (1976).

Adenylate cyclase assay

Adenylate cyclase activity was determined with the identical reaction mixture as described above for GTPase assay, with forskolin, 3-isobutyl-1-methylxanthine and cyclic AMP included, but instead of [γ - ^{32}P]GTP, [α - ^{32}P]ATP (0.2 μCi /tube) was present. Preincubation and incubation conditions were as for GTPase assay. The reaction was stopped and cyclic AMP formed was isolated as described by Jakobs *et al.* (1976). Both types of assays were performed in triplicate, with intra-assay variations of less than 3% of the means, and were repeated at least twice, with results comparable with those shown herein.

RESULTS

As reported previously (Aktories & Jakobs, 1984), thrombin decreased adenylate cyclase activity in membranes of human platelets more efficiently than did adrenaline (Fig. 1). When thrombin was combined with adrenaline at a maximally effective concentration (100 μM), the effects of both agents were not additive. Similar to adenylate cyclase inhibition, thrombin was more effective than adrenaline in stimulating GTP hydrolysis by high-affinity GTPase(s) in platelet membranes. However, in contrast with adenylate cyclase inhibition, the combined effects of adrenaline and thrombin on GTP hydrolysis were at least partially additive. These data suggested that thrombin activation of GTP hydrolysis in human platelet membranes is

Fig. 1. Interactions of adrenaline and thrombin on adenylate cyclase and high-affinity GTPase(s) in membranes of human platelets

Activities of adenylate cyclase (left ordinate: ○, □) and GTPase (right ordinate; ●, ■) were determined in membranes of human platelets in the absence (○, ●) and presence of 100 μM -adrenaline (□, ■) at the indicated concentrations of thrombin.

at least in part mediated by a GTPase not affected by agonist-stimulated α_2 -adrenoceptors. Therefore we studied whether the stimulation of GTP hydrolysis by thrombin is sensitive to agents causing inactivation of N_i . Treatment of the platelet membranes with pertussis toxin has been shown previously to inhibit considerably GTP hydrolysis caused by adrenaline (Aktories *et al.*, 1983). However, the thrombin stimulation of GTP hydrolysis was only partially decreased by this treatment (Table 1). Even at a concentration of 50 μg of pertussis toxin/ml, the absolute increase in GTP hydrolysis by high-affinity GTPase caused by thrombin (0.1 unit/ml) was decreased by only 25%, whereas adrenaline (100 μM)-induced stimulation of GTP hydrolysis was decreased by about

Table 1. Influence of pertussis-toxin treatment on stimulation of GTP hydrolysis by adrenaline and thrombin in membranes of human platelets

Membranes of human platelets were pretreated without (Control) and with 50 μg of pertussis toxin/ml for 30 min at 37 °C as described by Aktories *et al.* (1983). In these membranes, high-affinity GTPase activities were determined without and with 100 μM -adrenaline or 0.1 unit of thrombin/ml. The data (means $\pm$ S.E.M. for three experiments performed in triplicate) are the agonist-induced increase in GTPase activity.

Agonist	Increase in GTPase activity (pmol of P_i /min per mg of protein)	
	Control	Pertussis toxin
Adrenaline	6.2 $\pm$ 0.3	1.0 $\pm$ 0.2
Thrombin	10.8 $\pm$ 0.4	8.0 $\pm$ 0.2

Fig. 2. Influence of NEM on adrenaline- and thrombin-induced inhibition of platelet adenylate cyclase

Membranes of human platelets were pretreated for 15 min at 25 °C with the indicated concentrations of NEM. This reaction was stopped by adding 1 mM-dithiothreitol. Thereafter, adenylate cyclase activity was determined without (○) and with 100 μM-adrenaline (■) or 0.1 unit of thrombin/ml (●).

90% by this treatment, as described previously (Aktories *et al.*, 1983). Since pertussis toxin does not act on intact platelets (Ui *et al.*, 1984), about 1000-fold higher concentrations of the toxin than are usually applied in other intact cell systems had to be used with the platelet membranes.

The other agent that has been shown previously to eliminate N_1 -mediated actions in platelet membranes is the thiol reagent NEM (Jakobs *et al.*, 1982; Limbird & Speck, 1983). This agent additionally inactivates adenylate cyclase, apparently by a direct action at the catalytic moiety. As shown in Fig. 2, treatment of platelet membranes with NEM decreased and finally blocked agonist-induced adenylate cyclase inhibition. Whereas, in membranes pretreated without NEM, adrenaline (100 μM) and thrombin (0.1 unit/ml) decreased enzyme activity by about 50 and 70% respectively, the inhibition was decreased by treatment of the membranes with 30 μM-NEM to about 5 and 15% respectively, and was abolished after treatment with 100 μM or 300 μM-NEM. At the last two concentrations, NEM also decreased control adenylate cyclase activity. There was no principal difference between adrenaline and thrombin with regard to blockade of adenylate cyclase inhibition after NEM treatment. However, when the effect of NEM on GTP hydrolysis was studied, a marked difference between adrenaline and thrombin was revealed. Similar to adenylate cyclase inhibition, treatment of the membranes with NEM at increasing concentrations largely eliminated stimulation of GTP hydrolysis by adrenaline (Fig. 3). Already after treatment with 30 μM-NEM, the stimulatory effect of adrenaline was almost abolished. NEM treatment also decreased the increase in GTPase activity by thrombin by about 30% at 30 μM. However, in con-

Fig. 3. Influence of NEM on adrenaline- and thrombin-induced stimulation of GTP hydrolysis in platelet membrane

Membranes of human platelets were pretreated for 15 min at 25 °C with the indicated concentrations of NEM. This reaction was stopped by adding 1 mM-dithiothreitol. Thereafter, high-affinity GTPase activity was determined without (○) and with 100 μM-adrenaline (■) or 0.1 unit of thrombin/ml (●).

trast with adrenaline-induced stimulation, increasing the NEM concentration 10-fold (to 300 μM) did not cause a further decrease in thrombin stimulation of GTP hydrolysis, although basal unstimulated activity was decreased by the NEM treatment. Even after treatment with 3 mM-NEM, the stimulation of GTP hydrolysis by thrombin was essentially not changed further (results not shown).

Fig. 4 illustrates the concentration/response curves for adrenaline and thrombin stimulation of GTP hydrolysis in control membranes and membranes pre-treated for 15 min at 25 °C with 0.1 mM-NEM. In control membranes, thrombin stimulated GTP hydrolysis more efficiently than adrenaline, with half-maximal and maximal increase occurring at about 0.004 and 0.1 unit of thrombin/ml respectively. In NEM-pretreated membranes, the potency of thrombin in stimulating GTP hydrolysis was only slightly lessened. Half-maximal stimulation was observed at 0.006 unit of thrombin/ml. The maximal increase in GTP hydrolysis by thrombin was decreased by about 40% after the NEM treatment. On the other hand, adrenaline stimulated GTP hydrolysis in control membranes, with half-maximal and maximal activation at 1.5 μM and 30 μM respectively. This stimulatory effect of adrenaline was decreased by about 90% after NEM treatment. The potency of adrenaline seemed not to be changed by the NEM treatment, although, because of the small increase, an exact estimate cannot be given. Similar data with regard to the potency of thrombin and the decreased thrombin stimulation of GTP hydrolysis were obtained when the membranes were pretreated with 1 mM-NEM, as illustrated in Fig. 5. At the high NEM concentration used (1 mM), only basal unstimulated GTP hydrolysis was further decreased, whereas the increase by thrombin was identical with that obtained with the 10-fold lower concentration of NEM.

Fig. 4. Comparison of adrenaline- and thrombin-induced GTPase stimulations in control and NEM-pretreated platelet membranes

Membranes of human platelets were pretreated for 15 min at 25 °C without (○, □) or with 100 μM-NEM (●, ■). This reaction was stopped by adding 1 mM-dithiothreitol. Thereafter, high-affinity GTPase activities were determined at the indicated concentrations of adrenaline (□, ■) or thrombin (○, ●). GTPase activities of control and NEM-treated membranes are given on the left and right ordinates respectively.

Fig. 5. Stimulation of GTP hydrolysis by thrombin in platelet membranes pretreated with various concentrations of NEM

Membranes of human platelets were pretreated without (○) and with (●) 0.1 mM- or (■) 1 mM-NEM for 15 min at 25 °C. This reaction was stopped by adding 3 mM-dithiothreitol. Thereafter, high-affinity GTPase activity was determined at the indicated concentrations of thrombin.

In order to determine whether after the NEM treatment the substrate characteristics of the thrombin-stimulated GTP hydrolysis are changed, we studied the substrate kinetics of the GTPase reactions. Under all of the conditions studied, double-reciprocal plots of GTPase activity versus GTP concentration yielded straight lines (results not shown). In three separate experiments, the K_m values for GTP in control membranes determined in the absence and presence of thrombin (0.1 unit/ml) were $0.31 \pm 0.04 \mu\text{M}$ and $0.24 \pm 0.04 \mu\text{M}$ respectively. In membranes pretreated with 0.1 mM, the corresponding K_m values were 0.30 ± 0.05 and $0.23 \pm 0.03 \mu\text{M}$ respectively. The increases in V_{max} caused by thrombin were 15.9 ± 2.1 and 10.0 ± 1.2 pmol of P_i /min per mg of protein in control and NEM-treated membranes respectively.

The NEM resistance of thrombin stimulation of GTP hydrolysis by high-affinity GTPase in platelet membranes is similar to the NEM resistance of GTPase stimulation caused by PGE_1 , which via N_s activates adenylate cyclase in these membranes (Jakobs *et al.*, 1982; Lester *et al.*, 1982). In contrast with adrenaline-induced GTPase stimulation, the PGE_1 stimulation of GTP hydrolysis is impaired by treatment of the membranes with cholera toxin (Aktories *et al.*, 1983). Therefore we studied whether the thrombin stimulation of GTP hydrolysis may be affected by cholera toxin. Treatment of platelet membranes for 10 min at 30 °C with cholera toxin (100 μg/ml) decreased the increase in GTPase activity caused by PGE_1 (10 μM) from 5.0 ± 0.8 to 0.4 ± 0.2 pmol of P_i /min per mg of protein ($n = 3$). In contrast, both adrenaline (100 μM)- and thrombin (0.1 unit/ml)-induced increases in GTP hydrolysis were unaffected by this cholera-toxin treatment (results not shown).

DISCUSSION

Guanine nucleotide-binding regulatory proteins are apparently involved in the coupling of membrane-bound hormone receptors to their effector systems, which catalyse the formation of intracellular second messengers (Rodbell, 1980). Three of these proteins, namely N_s , N_i and transducin, have been identified, purified and at least partially characterized with regard to their functions in the signal-transduction processes. N_s and N_i function as coupling components of stimulatory and inhibitory hormone receptors, respectively, to the adenylate cyclase, and transducin couples light-activated rhodopsin to a cyclic GMP phosphodiesterase in rod outer segments (Kühn, 1980; Brandt *et al.*, 1983; Gilman, 1984; Bokoch *et al.*, 1984; Codina *et al.*, 1984; Sunyer *et al.*, 1984; Jakobs *et al.*, 1985). Another guanine nucleotide-binding protein (N_0) has been purified and biochemically characterized, but its function is not known so far (Sternweis & Robishaw, 1984; Neer *et al.*, 1984). All of these N proteins not only bind guanine nucleotides but also possess GTP-hydrolysing activity. On the other hand, evidence has now accumulated which suggests that the breakdown of membrane phosphoinositides caused by agonist-activated receptors also involves the coupling of a guanine nucleotide-binding regulatory protein (Litosch *et al.*, 1985; Cockcroft & Gomperts, 1985; Smith *et al.*, 1985; Lucas *et al.*, 1985). However, this protein has not been identified and purified so far. In some, but not all, cellular systems, studies with pertussis toxin, which has been shown to ADP-ribosylate and inactivate N_i (Ui

et al., 1984), suggested that a protein similar to N_i is involved in these cell types in the receptor-induced phosphoinositide breakdown (Bokoch & Gilman, 1984; Nakamura & Ui, 1985; Volpi *et al.*, 1985; Masters *et al.*, 1985).

In human platelets, thrombin is a potent and efficient activator of the phosphoinositide phosphodiesterase (Agranoff *et al.*, 1983; Siess *et al.*, 1984; Rittenhouse & Sasson, 1985). Functional studies performed in permeabilized platelets suggested that this apparently receptor-mediated action of thrombin involves the coupling function of a guanine nucleotide-binding component (Haslam & Davidson, 1984a,b). On the other hand, thrombin has been shown to induce adenylate cyclase inhibition in membranes of human platelets (Aktories & Jakobs, 1984). Similar to the platelet adenylate cyclase inhibition caused by agonist-activated α_2 -adrenoceptors, the thrombin-induced inhibition apparently involves the coupling of the inhibitory guanine nucleotide-binding regulatory protein N_i . Furthermore, similar to adrenaline, thrombin increases GTP hydrolysis in platelet membranes by activating a high-affinity GTPase(s) at the same concentrations as cause adenylate cyclase inhibition (Aktories & Jakobs, 1984). Therefore, it was assumed that, similar to adrenaline, the thrombin-induced stimulation of GTP hydrolysis is an action of N_i .

However, as shown herein, the thrombin-induced stimulation of GTP hydrolysis in platelet membranes can be discriminated from the GTPase stimulation by adrenaline and from the thrombin-induced adenylate cyclase inhibition, both reactions involving N_i . Firstly, whereas adrenaline and thrombin at maximally effective concentrations were not additive with regard to adenylate cyclase inhibition, the stimulation of GTP hydrolysis caused by these agents was at least partially additive. Secondly, treatment of the platelet membranes with pertussis toxin blocked adrenaline- and thrombin-induced adenylate cyclase inhibition and adrenaline-induced GTPase stimulation, whereas thrombin-induced stimulation of GTP hydrolysis was rather resistant to this treatment. Thirdly, treatment of the platelet membranes with NEM abolished adrenaline- and thrombin-induced adenylate cyclase inhibition at very similar concentrations of the thiol reagent. However, the NEM treatment eliminated almost completely only the adrenaline-induced GTPase stimulation, whereas the thrombin-induced increase in GTP hydrolysis was only partially decreased by this treatment, even at high NEM concentrations. The NEM and pertussis-toxin treatment decreased the thrombin-induced increase in GTP hydrolysis by high-affinity GTPase to a similar extent of 25–40%. All of these data suggest that thrombin activates in membranes of human platelets two GTP-hydrolysing enzymes, one of them apparently being N_i . Since the partial NEM resistance of the thrombin-induced GTPase stimulation is similar to the NEM resistance of GTPase activity of N_s stimulated by PGE_1 , we examined whether cholera-toxin treatment, which inhibits GTPase stimulation of N_s (Cassel & Selinger, 1977), would affect thrombin-induced GTPase stimulation. However, this was not the case, thus excluding the possibility that thrombin activates both N_i and N_s in platelet membranes.

After elimination of the N_i GTPase by NEM treatment, the remaining thrombin-induced stimulation of GTP hydrolysis exhibited characteristics quite similar

to those observed in untreated membranes. Firstly, the concentrations of thrombin causing half-maximal stimulation of GTP hydrolysis were very similar, 0.004 and 0.006 unit/ml in control and NEM-treated membranes respectively. Secondly, the K_m values of the thrombin-stimulated GTPase enzymes were identical, 0.2–0.3 μM -GTP, in control and NEM-treated membranes. These data do not contradict the assumption that thrombin activates two GTP-hydrolysing enzymes. Firstly, when studied in intact membranes, the N_s - and N_i -related GTP-hydrolysing enzymes exhibit almost identical K_m values in the range of 0.1–0.3 μM -GTP (Cassel & Selinger, 1976; Koski & Klee, 1981; Aktories & Jakobs, 1981). Furthermore, purified N_o GTPase has a K_m of about 0.3 μM (Neer *et al.*, 1984). It is thus feasible that the unknown GTP-binding protein activated by thrombin exhibits a similar K_m value for GTP to that of the purified N_s , N_i and N_o proteins. Secondly, the similarity in the potency of thrombin to activate the two apparently distinct GTP-hydrolysing enzymes may be due to the fact that the activated thrombin receptor interacts with the two N proteins with similar efficacy, or that there are two distinct receptor sites being activated by thrombin at very similar concentrations. At present, none of these possibilities can be excluded, since there are no specific tools to activate one or the other putative thrombin receptor or to inactivate selectively the GTPase activity apparently not related to N_i .

With regard to the possible function of the pertussis-toxin- and NEM-resistant GTP-hydrolysing enzyme activated by thrombin, it is tempting to speculate that this component is involved in the coupling of thrombin receptors to the phosphoinositide phosphodiesterase. According to the data presented herein, this thrombin action should be insensitive to pertussis-toxin and NEM treatment, provided that these agents do not have additional, as yet unknown, effects on the phosphoinositide phosphodiesterase. Studies on the regulation of this signal transduction in platelet membranes are needed to understand the underlying coupling mechanisms.

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