Stimulation of high-affinity GTPase by trypsin and trypsin-like proteinases in membranes of human platelets

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The influence of various proteinases on GTP hydrolysis was studied in membranes of human platelets. Of the proteinases examined, trypsin, acrosin and a recently described trypsin-like proteinase from bovine sperm, but not chymotrypsin, increased GTP hydrolysis. Similar to what was described previously for hormone-like agents, the stimulation of GTP hydrolysis by the proteinases was only observed at low GTP concentrations, with apparent K_m values of 0.2–0.3 μM -GTP. Stimulation of the high-affinity GTPase by the proteinases occurred without apparent lag phase and was constant over a long period of incubation. The proteinase inhibitors leupeptin and soya-bean trypsin inhibitor blocked the stimulation of GTP hydrolysis, but did not reverse the effect of the proteinases. Treatment of platelet membranes with *N*-ethylmaleimide, which eliminates G_i -protein (inhibitory guanine-nucleotide-binding protein)-related GTPase stimulation by adrenaline, decreased stimulation of GTP hydrolysis by the proteinases only partially. Activation of GTP hydrolysis by the proteinases was partially additive with that caused by adrenaline, whereas thrombin stimulation was not increased further. The data indicate that, similarly to the proteinase thrombin, trypsin and trypsin-like proteinases can activate GTP-hydrolysing protein(s) that exhibit high affinity for GTP in platelet membranes. It is suggested that the proteinases interact in platelet membranes with a receptor site similar to that used by thrombin and that the observed GTPase stimulation is a reflection of a proteinase-receptor interaction with a guanine-nucleotide-binding regulatory protein.

INTRODUCTION

Thrombin is one of the most potent and efficient platelet-activating agents. Its action on platelets exhibits characteristics of an enzymic reaction as well as of an agonist–receptor action (Martin *et al.*, 1975; Mills & Macfarlane, 1976). At least two hormone-like actions of thrombin have been reported so far in platelets. First, as shown by many laboratories, thrombin can stimulate the hydrolysis of platelet polyphosphoinositides, leading to the formation of inositol phosphates and 1,2-diacylglycerol (Agranoff *et al.*, 1983; Siess *et al.*, 1984; Rittenhouse & Sasson, 1985). These two products are thought to mediate, at least in part, the cellular actions of thrombin. Functional studies performed on permeabilized platelets and platelet membranes suggest that the stimulatory action of thrombin on platelet phospholipase C, cleaving the phosphoinositides, may involve a guanine-nucleotide-binding regulatory protein, which, however, has not been identified or functionally characterized so far (Haslam & Davidson, 1984a,b; Baldassare & Fisher, 1986). A second hormone-like action of thrombin has been observed by studies in platelet membranes. Here it was shown that thrombin can potently and efficiently inhibit cyclic AMP formation by adenylate cyclase (Aktories & Jakobs, 1984). Evidence has been presented that this inhibition is mediated by the guanine-nucleotide-binding regulatory protein G_i , which couples inhibitory hormone receptors to adenylate cyclase. Furthermore, similar to various other hormonal

factors, thrombin stimulates GTP hydrolysis in platelet membranes by activating high-affinity GTPases (Aktories & Jakobs, 1984; Grandt *et al.*, 1986).

Thrombin is not unique as a proteolytic enzyme capable of activating platelets. Platelet secretion and aggregation has also been observed on addition of other proteinases, such as trypsin, papain and Pronase (Davey & Lüscher, 1967; Martin *et al.*, 1975). On the other hand, chymotrypsin has been found to be ineffective in this regard. It has been shown that, similarly to thrombin, trypsin can also lead to a stimulation of phosphoinositide breakdown in intact human platelets (Ruggiero & Lapetina, 1985). Furthermore, the serine-proteinase inhibitor leupeptin was found to inhibit the actions of both thrombin and trypsin. On the basis of these data, a direct proteolytic activation of phospholipase C was suggested as a possible mechanism of thrombin and trypsin action in platelets (Ruggiero & Lapetina, 1985).

One of the first events in many receptor–effector coupling systems appears to be the interaction of the agonist-activated receptor with a guanine-nucleotide-binding regulatory protein (Rodbell, 1980). This interaction can be monitored as an agonist-induced stimulation of GTP hydrolysis by a high-affinity GTPase activity of the G-protein, apparently reflecting the receptor-stimulated turnover of the G-protein from a GDP-bound to a GTP-bound state, with subsequent hydrolysis of bound GTP to GDP. Therefore we investigated whether, similarly to thrombin, other pro-

Abbreviations used: G_i -protein, the guanine-nucleotide-binding regulatory protein that couples inhibitory hormone receptors to adenylate cyclase; NEM, *N*-ethylmaleimide; TPCK, *N*-tosyl-L-phenylalanylchloromethane.

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Fig. 1. Influence of various proteinases on GTP hydrolysis in human platelet membranes

High-affinity GTPase activity was determined in membranes of human platelets at the indicated concentrations of (a) trypsin (○), TPCK-trypsin (●) and α -chymotrypsin (■), (b) purified boar acrosin and (c) crude bovine sperm proteinase preparation.

teolytic enzymes may also activate a high-affinity GTPase. We report here that trypsin and some other trypsin-like proteinases stimulate GTP hydrolysis by high-affinity GTPase(s) in membranes of human platelets.

MATERIALS AND METHODS

Materials

Trypsin, α -chymotrypsin, TPCK-trypsin (i.e. trypsin treated with TPCK to inhibit residual chymotryptic activity), leupeptin and soya-bean trypsin inhibitor were obtained from Sigma Chemical Co. Acrosin, purified from boar sperm, was kindly donated by Dr. H. Fritz, München, Germany. A proteinase with trypsin-like characteristics, but apparently not acrosin, was extracted from bovine sperm particles as described by Johnson *et al.* (1985). The preparation was used after extraction of the sperm particles with high salt (500 mM-NaCl) without further purification. All other agents used were obtained from sources previously described (Jakobs *et al.*, 1982; Aktories *et al.*, 1983; Aktories & Jakobs, 1984).

Preparation 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). The membranes were kept frozen in small batches at -70°C and thawed immediately before GTPase assay.

GTPase assay

GTPase activity in membranes of human platelets was determined with a reaction mixture containing, if not otherwise indicated, $0.5\ \mu\text{M}$ - $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ ($0.1\ \mu\text{Ci}/\text{tube}$), $100\ \mu\text{M}$ -ATP, 2 mM-MgCl₂, 0.1 mM-EGTA, 1 mM-dithiothreitol, 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 $100\ \mu\text{l}$. The reaction was started by the addition of platelet membranes (5–12 μg of protein/tube) and conducted for 10 min at 25°C . Termination of the reaction and isolation of $[\text{P}_i]$

formed were carried out as previously described (Aktories *et al.*, 1983). Low-affinity GTPase activity was determined and subtracted from the total GTPase activity by measuring the GTP hydrolysis at $50\ \mu\text{M}$ -GTP as described by Cassel & Selinger (1976). Less than 10 % of total GTP hydrolysis was due to low-affinity GTPase(s) under the assay conditions used. The assays were performed in triplicate with an intra-assay variation of about 2 %. Results similar to those shown herein were obtained in at least two additional experiments.

RESULTS

When the effects of α -chymotrypsin, trypsin and trypsin treated with TPCK (TPCK-trypsin) were studied on GTP hydrolysis in human platelet membranes measured at 25°C with $0.5\ \mu\text{M}$ -GTP as substrate, only trypsin and TPCK-trypsin significantly increased GTP hydrolysis (Fig. 1a). Maximal and half-maximal increases were observed at about 20 and 100 ng of either trypsin form/ml respectively. In contrast, α -chymotrypsin had no significant effect up to 100 ng/ml and decreased GTP hydrolysis at higher concentrations. This decrease was also observed with trypsin (200 ng/ml), but not with TPCK-trypsin at this concentration. When studies similar to those in Fig. 1(a) were performed at 30°C instead of 25°C , the inhibitory phases of trypsin and chymotrypsin actions on GTP hydrolysis were observed at about 2-fold lower concentrations of these proteinases, whereas the maximal increase in GTP hydrolysis and the EC_{50} value (concn. giving half-maximal effect) of trypsin for stimulation of GTPase were not significantly changed (results not shown). The trypsin-like proteinase acrosin purified from boar sperm (Müller-Esterl & Fritz, 1981) increased GTP hydrolysis to a similar extent to trypsin and TPCK-trypsin (Fig. 1b). However, the potency of acrosin in increasing GTP hydrolysis was about 100-fold lower than that of trypsin; half-maximal increase in GTP hydrolysis was observed at $1.5\ \mu\text{g}/\text{ml}$. A third trypsin-like proteinase, previously described in high-salt extracts of bovine sperm particles (Johnson *et al.*, 1985), increased GTP hydrolysis to a similar extent as did trypsin and

Fig. 2. Time course of GTP hydrolysis in platelet membranes: influence of various proteinases

GTP hydrolysis by high-affinity GTPase was studied in membranes of human platelets for the indicated periods of time at 30 °C in the absence (○) and the presence of α -chymotrypsin (Δ ; 100 ng/ml), trypsin (●; 50 ng/ml) or the crude bovine sperm proteinase preparation (■; 1 μ l/tube).

acrosin (Fig. 1c). Since a crude preparation was used, no estimate of the potency of this proteinase can be given. All the proteinases studied exhibited a clear maximal effect.

The stimulatory effects of trypsin and the bovine sperm proteinase on GTP hydrolysis in platelet membranes occurred without apparent lag phase (Fig. 2). Furthermore, the stimulation was not changed in its extent, neither a further increase nor a decrease being observed during the incubation period studied. Even when the incubation was performed for up to 30 min, the stimulatory effect of the proteinases was not altered. Furthermore, similar time relationships were observed at incubation temperatures of 25 and 30 °C (results not shown). The time-course experiment additionally shows that α -chymotrypsin did not increase GTP hydrolysis, even when measured at early time points. When the GTPase activity was studied with 30 μ M- or 50 μ M-GTP as substrate, none of the proteinases studied significantly increased GTP hydrolysis. Therefore the influence of the proteinases on the GTP hydrolysis reaction was measured at various low substrate concentrations. As illustrated in Fig. 3, the bovine sperm proteinase increased GTP hydrolysis at any low GTP concentration studied. The V_{max} of the reaction was increased by 40–60 % without major change in the apparent K_m value for GTP, which was $0.23 \pm 0.04 \mu$ M and $0.28 \pm 0.03 \mu$ M ($n = 3$) in the absence and the presence of the sperm proteinase respectively. Similar data were obtained with trypsin and acrosin (results not shown).

The increase in GTP hydrolysis in platelet membranes by trypsin and the bovine sperm proteinase was completely blocked by the proteinase inhibitor leupeptin (10 μ g/ml) when added together with the proteinases

Fig. 3. Influence of the bovine sperm proteinase on substrate kinetics of platelet membrane high-affinity GTPase

GTP hydrolysis was studied in membranes of human platelets in the absence (○) and the presence (●) of the crude bovine sperm proteinase preparation (1 μ l/tube) at the indicated concentrations of GTP.

Table 1. Influence of leupeptin on platelet high-affinity-GTPase stimulation by proteinases

High-affinity GTPase activity was determined in membranes of human platelets in the absence and presence of trypsin (50 ng/ml) or the crude bovine sperm proteinase preparation (1 μ l/tube) without or with leupeptin (10 μ g/ml) present. 'No preincubation': leupeptin was added together with the proteinases; 'preincubation': the membranes were first preincubated for 10 min at 25 °C without and with the proteinases, followed by the addition of leupeptin and, thereafter, of labelled GTP to start the GTPase reaction. The results are means $\pm$ S.E.M. ($n = 3$).

Addition	GTPase activity (pmol of P_i /min per mg of protein)		
	Control	+ Trypsin	+ Sperm proteinase
No preincubation			
None	22.3 $\pm$ 0.4	29.1 $\pm$ 0.3	36.4 $\pm$ 0.5
Leupeptin	21.4 $\pm$ 0.2	21.1 $\pm$ 0.4	21.5 $\pm$ 0.3
Preincubation			
None	12.4 $\pm$ 0.3	16.1 $\pm$ 0.4	19.2 $\pm$ 0.5
Leupeptin	12.5 $\pm$ 0.2	16.0 $\pm$ 0.3	19.3 $\pm$ 0.4

(Table 1). However, when the platelet membranes were pretreated with the proteinases for 10 min at 25 °C, addition of the proteinase inhibitor did not reverse the proteinase-induced increase in GTP hydrolysis. The preincubation by itself, i.e. without proteinases, caused a decrease in GTPase activity, which was not affected by leupeptin. Similar results to those with leupeptin were obtained with soya-bean trypsin inhibitor (not shown).

To gain some insight into the nature of the guanine-nucleotide-binding protein affected by the proteinases, we studied the influence of *N*-ethylmaleimide (NEM)

Fig. 4. Influence of NEM on GTPase activation in platelet membranes by various agents

Membranes of human platelets were pretreated for 10 min at 25 °C with the indicated concentrations of NEM. This reaction was stopped by adding 500 μM-dithiothreitol. Thereafter, high-affinity GTPase activity was determined without (○) or with 100 μM-adrenaline (□), 0.1 unit of thrombin/ml (■) or 50 ng of trypsin/ml (●).

Table 2. Influence of the bovine sperm proteinase on stimulation of platelet high-affinity GTPase by adrenaline and thrombin

High-affinity GTPase activity was determined in membranes of human platelets without and with the crude bovine sperm proteinase preparation (1 μl/tube) in the absence and the presence of adrenaline (30 μM) or thrombin (0.1 unit/ml). 'No preincubation': the sperm proteinase was added together with adrenaline or thrombin; 'preincubation': the membranes were first preincubated for 10 min at 25 °C without and with the sperm proteinase, followed by addition of leupeptin (10 μg/ml) and, thereafter, of adrenaline and thrombin. Data are means ± S.E.M. (n = 3).

Addition	GTPase activity (pmol of P _i /min per mg of protein)		
	Control	+ Adrenaline	+ Thrombin
No preincubation			
None	18.1 ± 0.5	24.6 ± 0.4	35.5 ± 0.6
Sperm proteinase	25.8 ± 0.4	32.6 ± 0.6	33.6 ± 0.5
Preincubation			
None	10.0 ± 0.2	15.8 ± 0.4	21.4 ± 0.4
Sperm proteinase	15.4 ± 0.2	20.6 ± 0.3	18.6 ± 0.2

on GTP hydrolysis. Pretreatment of intact platelets or platelet membranes with this agent has been shown to block adrenaline-induced GTPase stimulation in platelet membranes (Jakobs *et al.*, 1982). As shown in Fig. 4, pretreatment of platelet membranes for 10 min at 25 °C with NEM abolished GTPase stimulation caused by the α₂-adrenoceptor-agonist adrenaline (100 μM). In con-

trast, GTPase stimulation by trypsin (50 ng/ml), which increased GTP hydrolysis to a smaller extent than with adrenaline in untreated membranes, was only partially decreased by the NEM treatment. After treatment with 300 μM-NEM, which virtually eliminated adrenaline-induced stimulation, about 50 % of stimulation by trypsin and bovine sperm proteinase (results not shown) of GTP hydrolysis was still preserved. Similar data were obtained with thrombin (0.1 unit/ml), which caused a much larger increase in GTP hydrolysis than either adrenaline or trypsin, and which stimulation was also only partially decreased by the NEM treatment (Grandt *et al.*, 1986). Additional support for different GTPases being activated by adrenaline and the proteinases was obtained when the question of the additivity was studied (Table 2). When adrenaline at a maximally effective concentration (30 μM) was combined with the bovine sperm proteinase or trypsin (results not shown), or when adrenaline was added to platelet membranes pretreated with the proteinase and thus exhibiting persistently increased GTPase activity, almost additive effects of adrenaline and the proteinases were obtained. In contrast, there was no additivity, but usually a decrease, when the combined effects of thrombin and the proteinases were studied.

DISCUSSION

The data presented in this report indicate that trypsin and trypsin-like proteinases, but not α-chymotrypsin, can stimulate GTP hydrolysis in membranes of human platelets, in a manner similar to that described previously for the proteinase thrombin (Aktories & Jakobs, 1984; Grandt *et al.*, 1986). Both types of proteinases only increased GTP hydrolysis caused by high-affinity GTPase(s), with apparent K_m values of 0.2–0.3 μM-GTP, whereas low-affinity GTPase activities were not increased. Furthermore, the stimulatory effect of the proteinases on GTP hydrolysis occurred without apparent lag phase. Additionally, the stimulation was constant over a rather long period of time, without a decrease or a further increase being observed during the incubation. All of these data suggest that these proteinases, including thrombin, induce stimulation of GTP hydrolysis in a manner similar to that caused by hormonal factors acting via well-characterized cell-surface receptors. On the other hand, as also observed with thrombin (Aktories & Jakobs, 1984), agents that inhibit the proteolytic activity of these enzymes, such as leupeptin and hirudin, prevented the stimulation of GTP hydrolysis when added together with the proteinases. However, when the reaction was initiated by the proteinases, subsequent addition of the proteinase inhibitors did not reverse the stimulation of GTP hydrolysis. These data suggest, as proposed previously for stimulation of intact platelets by thrombin and other proteinases (Martin *et al.*, 1975), that the activation of platelet membrane high-affinity GTPase by thrombin and the proteinases studied herein may involve a catalytic step at the receptor site, which reaction can be prevented by simultaneous addition of proteinase inhibitors, with subsequently no dissociation of the enzyme (agonist) from its modified product (receptor), which reaction is then not susceptible to agents such as leupeptin and hirudin. That the stimulation of GTP hydrolysis by the proteinases is most probably not due to a direct action at the GTP-hydrolysing protein(s), but is mediated through

a distinct receptor site, is corroborated by the finding that these proteinases increased GTP hydrolysis in membranes of human platelets, but not in membranes of S49 lymphoma cyc^- cells and neuroblastoma $\times$ glioma hybrid cells (NG 108-15) (results not shown).

The observation that trypsin and trypsin-like proteinases, but not α -chymotrypsin, increased GTP hydrolysis by high-affinity GTPase(s) in platelet membranes is in agreement with data obtained in intact platelets. Only trypsin and trypsin-like proteinases, but not α -chymotrypsin, induce rapid platelet activation, including stimulation of phosphoinositide metabolism, secretion and aggregation (Davey & Lüscher, 1967; Martin *et al.*, 1975; Ruggiero & Lapetina, 1985). These data obtained on intact platelets additionally suggest that the action of these proteinases on platelets is caused by an interaction with a structure located at the outer surface of the plasma membrane, such as a receptor protein. This protein, when affected by the proteinases, appears to induce the observed subsequent functional changes, such as stimulation of GTP hydrolysis and activation of the phosphoinositide metabolism.

With regard to the nature of the proteins that hydrolyse GTP with high affinity and that are affected by the proteinases, the additivity data with other platelet agonists and the data obtained by NEM treatment of platelet membranes suggest that the GTPases stimulated by the proteinases are similar to or identical with those affected by thrombin. Previous data with pertussis toxin and NEM, which can both inactivate the G_i -protein, suggest that thrombin stimulation of GTP hydrolysis in platelet membranes is due to two distinct high-affinity GTPases (Grandt *et al.*, 1986; Houslay *et al.*, 1986). As also shown herein (Fig. 4), treatment of platelet membranes with NEM eliminated stimulation of GTP hydrolysis by adrenaline, which after interaction with α_2 -adrenoceptors activates G_i , with subsequent inhibition of adenylate cyclase. In contrast, the stimulatory actions of the proteinases, including that of thrombin, were only partially decreased by the NEM treatment. On the other hand, as shown previously (Aktories & Jakobs, 1984; Grandt *et al.*, 1986), NEM at rather low concentrations abolishes activation of G_i by thrombin, with subsequent inhibition of adenylate cyclase. Therefore it is proposed that thrombin and the proteinases studied herein may interact with one common or two distinct receptor sites in platelet membranes, and that this interaction leads to an activation of G_i and of an additional, not yet defined, guanine-nucleotide-binding component exhibiting GTP-hydrolysing activity. Although activated G_i can lead to

inhibition of adenylate cyclase, the other putative guanine-nucleotide-binding protein may act as coupling component between receptors and phospholipase C. Studies of these possible functional consequences of G-protein activation by the proteinases are now required.

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