Thrombin inhibits the pertussis-toxin-dependent ADP-ribosylation of a novel soluble G_i -protein in human platelets

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A new G-protein was detected in human platelets which was ADP-ribosylated in a pertussis-toxin-dependent manner, was located in the supernatant of saponized platelets and was of a slightly lower molecular mass (40 kDa) than platelet membrane $G_{i\alpha}$. This soluble ADP-ribosylated protein was immunoprecipitated by an antiserum to $G_{i\alpha}$, but not by one to G_{α} . Prior thrombin stimulation of platelets led to an inhibition of the ADP-ribosylation of this protein. This inhibition was evident even under conditions which abolished the thrombin-stimulated inhibition of membrane $G_{i\alpha}$ ADP-ribosylation. These results indicate that the platelet thrombin receptor is coupled to two structurally and functionally distinct $G_{i\alpha}$ proteins: a major $G_{i\alpha}$ protein present in platelet membranes, and a minor $G_{i\alpha}$ protein detectable in the platelet soluble fraction.

INTRODUCTION

Guanine-nucleotide-binding proteins (G-proteins) are a growing family of regulatory proteins which couple membrane receptors to effector proteins which generate an intracellular response [1,2]. Activation of a heterotrimeric G-protein by an agonist-occupied receptor results in exchange of bound GDP for GTP in the α -subunit and dissociation of the α -subunit from the β, γ complex. At least nine different G_{α} proteins have been identified through protein purification or gene cloning [1–4]. Gene-cloning experiments have described three human $G_{i\alpha}$ (α_1 , -2 and -3) subunits with apparent molecular masses of 40–41 kDa, which show high similarity to each other. The inhibitory GTP-binding protein G_i mediates inhibition of adenylate cyclase in numerous cell types, including platelets [5–9]. At least two $G_{i\alpha}$ subunits (α_2 and α_3) are expressed in platelets [10–13]. Recently, $G_{i\alpha_2}$ has been shown to mediate α_2 -adrenergic inhibition of adenylate cyclase in platelets [12] and neuroblastoma cells [14]. All characterized $G_{i\alpha}$ subunits are ADP-ribosylated by pertussis toxin, but only if associated with β and γ subunits [2,15–17]. A G-protein of undefined function, G_o , is also a pertussis-toxin substrate, but little or none is present in platelets [11,12].

Thrombin is a potent platelet activator which stimulates aggregation and secretion, inhibits adenylate cyclase and induces phospholipase C activation, arachidonate release, protein kinase C activation and Ca^{2+} mobilization (reviewed in [18]). Detwiler and co-workers [19,20] have demonstrated that chymotrypsin treatment of intact platelets modifies differentially thrombin-stimulated responses. On the basis of that work, thrombin responses could be classified into two groups. Group 1 responses (adenylate cyclase inhibition and arachidonate release) are completely blocked, whereas group 2 responses (aggregation, phospholipase C and protein kinase C activation) are only slowed by chymotrypsin treatment. This led to the proposal that either two thrombin receptors are present on platelets or two distinct transducing and effector systems are coupled to one receptor. Two lines of evidence suggest that these responses are

coupled to the thrombin receptor by G_i -proteins. Thrombin inhibits adenylate cyclase in intact platelets and platelet membranes through a pertussis-toxin-sensitive G_i -protein [21,22]. Prior thrombin stimulation of intact platelets inhibits the subsequent pertussis-toxin-dependent ADP-ribosylation of membrane G_i [23]. G_i might also be involved in the thrombin-mediated stimulation of phospholipase C [24], but does not seem to activate the enzyme directly [25]. We describe here two distinct G_i proteins which are coupled to the platelet thrombin receptor. One is membrane-bound $G_{i\alpha}$, most probably identical with $G_{i\alpha_2}$ [10–13], the well-characterized substrate for pertussis toxin. The second is a G_i protein with a smaller α -subunit, which is ADP-ribosylated in a pertussis-toxin-dependent manner and is located in the supernatant of saponized platelets.

EXPERIMENTAL

Materials

Thrombin, adrenaline, vasopressin, Ca^{2+} ionophore A23187, pertussis toxin, cholera toxin, saponin, prostacyclin (PGI_2), apyrase, goat anti-(rabbit IgG)-alkaline phosphatase conjugate, chymotrypsin and leupeptin were obtained from Sigma Chemical Co. [$adenylate$ - ^{32}P]NAD $^{+}$ and [$adenosine$ -U- ^{14}C]NAD $^{+}$ were obtained from New England Nuclear (Boston, MA, U.S.A.). A mixture of purified bovine brain G_i and G_o [26] was kindly given by Dr. P. Gierschik (Pharmacology Institute, University of Heidelberg, Heidelberg, Germany), as was the affinity-purified antibody AS/6. This antibody recognizes the C-terminus of the α -subunit of transducin and G_i , but not G_o and G_s [27]. Antisera AS/7, LD/2 and GO/1 [9,27] were kindly supplied by Dr. A. Spiegel (NIH, Bethesda, MD, U.S.A.).

Platelet preparation

Platelets were isolated from freshly drawn whole blood essentially as described previously [29,30]. Typically, 100 ml of citrate-treated blood was centrifuged in a Kontron H-401 centrifuge with AS 44 rotor at 1000 rev./min for 15 min at 25 °C. The platelet-rich plasma was incubated with aspirin (1 mM) at 37 °C

Abbreviations used: PEG, polyethylene glycol; PGI_2 , prostaglandin I_2 (prostacyclin).

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for 15 min. Platelets were sedimented in the presence of 250 ng of PGI_2 /ml by centrifugation at 2500 rev./min for 10 min. The platelet pellet was washed once in buffer A containing 1 mM-EGTA, 250 ng of PGI_2 /ml and apyrase (0.5 unit of ADPase/ml). Buffer A consists of 20 mM-Hepes, 138 mM-NaCl, 2.9 mM-KCl, 1 mM- MgCl_2 , 5 mM-glucose (pH 7.4). Washed platelets were resuspended in 1.8 ml of buffer A [approx. $(1-3) \times 10^9$ cells/ml] and treated with 5 mM-EGTA at 37 °C for 15 min immediately before the start of each experiment.

Chymotrypsin treatment of intact platelets

Platelets were isolated as described above, but resuspended in 5 ml of buffer A (2×10^8 cells/ml). Platelet suspensions were incubated without addition or with 10 μg of chymotrypsin/ml at 25 °C for 5 min with gentle shaking [19,20]. At the end of the incubation period, 15 ml of buffer A containing 1 mM-EGTA and 250 ng of PGI_2 /ml was added and pellets were sedimented as described above. After two washes with buffer A supplemented with EGTA and PGI_2 , platelets were resuspended in 1 ml of buffer A and treated with EGTA as described above.

Platelet stimulation and ADP-ribosylation reactions

Platelet suspensions were stimulated by agonists while being stirred (1100 rev./min) in a LABOR-aggregometer at 37 °C. Samples (80 μl) of platelet suspensions were removed at the times indicated in the Figure legends and added to 20 μl of an ADP-ribosylation cocktail, consisting of 42 mM-thymidine, 4.2 mM-ATP, 4.2 mM-EDTA, 0.5 mM-GTP, 4.2 mM-NAD⁺, 0.53 mg of saponin/ml, 40 μg of pertussis toxin (preactivated with 1 mM-dithiothreitol at 37 °C for 30 min)/ml and 100 μM -[³²P]NAD⁺ (approx. 8.3 Ci/mmol). In some experiments pertussis toxin was omitted from the ADP-ribosylation cocktail. When cholera-toxin-dependent ADP-ribosylation was studied, the cocktail was modified by addition of 60 mM-potassium phosphate (pH 7), 100 mM- MgCl_2 and 144 μg of cholera toxin (preactivated as described above)/ml and the omission of pertussis toxin. The saponized platelet samples were incubated with ADP-ribosylation cocktail for 1 h at 37 °C with gentle shaking. After incubation, saponized platelets were fractionated by centrifugation for 5 min at room temperature at top speed in an Eppendorf 3200 microfuge. The supernatant fraction (80 μl) was immediately added to electrophoresis sample buffer (20 μl). After aspiration of the residual supernatant, electrophoresis sample buffer (20 μl) was added to the pellet fraction and incubated at 37 °C for 1 h. Electrophoresis sample buffer consisted of 156 mM-Tris/HCl (pH 6.8), 5% (v/v) SDS, 10% (v/v) 2-mercaptoethanol and 0.05% Bromophenol Blue. When pellets had dissolved, buffer A containing 5 mM-EGTA (80 μl) was added. All samples were boiled for 2 min before electrophoresis.

Electrophoresis, immunoblotting and autoradiography

SDS/PAGE was carried out in the horizontal LKB 2117 Multiphor II system from Pharmacia (Freiburg, Germany) or the vertical system (20 cm long) from Bio-Rad (Richmond, CA, U.S.A.). A 2 mm-thick 11%-polyacrylamide running gel with 5% stacking gel was utilized. After electrophoresis, the gel's dye front (containing [³²P]NAD⁺) and stacking gel were cut from the running gel and discarded. The running gel was then washed with water for 1 h to remove residual [³²P]NAD⁺ before Coomassie Blue staining. The destained gel was dried and, with use of intensifying screens, exposed to Kodak X-OMAT-AR2 film at room temperature for 24–48 h.

When immunoblotting was conducted, proteins were electrophoretically transferred from SDS/polyacrylamide gels to

nitrocellulose by using the PolyBlot Transfer System (ABN, Hayward, CA, U.S.A.) and the procedure recommended by the manufacturer. Blocking of residual sites on nitrocellulose was effected by incubating blots overnight at 4 °C with wash buffer [50 mM-Tris/HCl, 0.2 M-NaCl, 0.02% NaN_3 , 1 mg of BSA/ml and 1 mg of PEG (20 kDa)/ml] containing 3% BSA. The blocked blot was incubated with a 1:2000 dilution of the $\text{G}_i\alpha$ -specific antibody AS/6 or AS/7 in wash buffer for 1 h at room temperature. After three washes of the blot with water, antibody binding was coupled to the secondary antibody goat anti-rabbit IgG-alkaline phosphatase conjugate diluted 1:1000 in wash buffer, by incubation of the blot for 45 min at room temperature. After three washes of the blot, antibody binding was detected by incubating blots in 0.1 M-Tris/0.1 M-NaCl/5 mM- MgCl_2 (pH 9.5) containing 0.33 mg of NitroBlue Tetrazolium/ml and 0.17 mg of 5-bromo-4-chloro-3-indolyl phosphate/ml. Autoradiography of the immunoblot was carried out as described above. Protein bands of the dried gel or immunoblot were cut out and incorporated radioactivity was determined by liquid-scintillation counting.

For separate ADP-ribosylation of the platelet particulate and supernatant fractions, the procedure was as follows: EGTA (4 mM)-treated platelet suspensions were lysed in the presence of leupeptin (1 mM) by incubation with saponin (125 μg /ml) for 15 min at 37 °C. Platelet supernatant and particulate fractions were obtained by centrifugation at maximal speed in a microfuge for 5 min. Supernatant (70 μl) and particulate fractions (after resuspension in 70 μl of EGTA-containing buffer) were incubated with 20 μl of ADP-ribosylation cocktail (see above) for 1 h at 37 °C. Samples (50 μl) of the supernatant were then transferred into 1 ml of ice-cold 10% (w/v) trichloroacetic acid, and left on ice for 30 min. Samples were centrifuged at 3000 g for 15 min and the pellets were washed with 2×1 ml of water-saturated diethyl ether. Pellets were resuspended in 0.16 ml of sample buffer containing 1.5% SDS and dithiothreitol. ADP-ribosylated particulate fractions were pelleted by centrifugation in a microfuge for 5 min and resuspended in 0.1 ml of sample buffer. Proteins were separated on SDS/PAGE (10%) gels (20 cm long, 1.5 mm thick). The gels were dried and exposed to Kodak X-OMAT-AR2 or BB5 films at 80 °C by using cassettes with an intensifying screen.

Immunoprecipitation of $\text{G}_i\alpha$ from platelets

This was done by the methods described in ref. [30]. The following antisera [9,26,27] were used: an anti- $\text{G}_i\alpha$ common antiserum (AS/7), which was produced against the C-terminal decapeptide (KENLKDCGLF) of rod transducin; the antiserum anti- $\text{G}_o\alpha$ (GO/1), which was produced against an internal sequence of brain $\text{G}_o\alpha$; and the antiserum anti- $\text{G}_i\alpha$ -1 (LD/2), which was produced against an internal sequence (LDRIAQPNYI) of $\text{G}_i\alpha$ -1.

Protein A-Sepharose (200 mg) was coated by incubation with BSA (50 mg) in phosphate-buffered saline (150 mM-NaCl/50 mM- Na_2HPO_4 , pH 7.35) for 1 h at 4 °C. After centrifugation (3000 g for 15 min), Protein A-Sepharose was washed with 2×40 ml of ice-cold RIPA buffer (see below) and resuspended in 4 ml of RIPA buffer.

Samples (0.36 ml) of platelet suspensions (4.5×10^9 platelets/ml) were incubated with 90 μl of ADP-ribosylation cocktail for 1 h at 37 °C. Particulate and supernatant fractions were separated by centrifugation in a microfuge at maximal speed for 5 min. Supernatant (0.4 ml) and pellet (resuspended in 0.35 ml of 5 mM-EGTA-containing buffer A) fractions were solubilized with 0.4 ml of lysis buffer (pH 7.4) containing 1% SDS, aprotinin (20 μg /ml), NaCl (150 mM), Na_2HPO_4 (50 mM) and dithiothreitol (2 mM), and heated for 1 min at 90 °C. Then

80 μ l of 10 $\times$ RIPA (10% deoxycholate, 10% Triton X-100, 150 mM-NaCl and 50 mM- Na_2HPO_4) was added to clear the extract. Samples (0.4 ml) were incubated with 4 μ l of normal rabbit serum for 1 h at 4°C. Then Protein A-Sepharose (see above) coated with BSA (5 mg in 100 μ l of RIPA containing 1% deoxycholate and 1% Triton X-100) was added and incubated for 1 h on ice. After centrifugation in a microfuge for 5 min, the supernatant (0.45 ml) was incubated with 4 μ l of specific anti-serum for 1 h at 4°C, and then with 100 μ l of Protein A-Sepharose for 1 h at 4°C. After centrifugation, the supernatant was discarded and Protein A-Sepharose was washed with 3 $\times$ 1 ml of RIPA buffer and once with phosphate-buffered saline. The samples were resuspended in 100 μ l of electrophoresis sample buffer and treated for 3 min at 100°C. The supernatant was separated by SDS/PAGE (10% gels).

RESULTS

Immunoblot analysis of $G_{i\alpha}$ from thrombin-stimulated platelets

The inability of pertussis toxin to ADP-ribosylate G_i after thrombin stimulation is believed to be due to a receptor-mediated G-protein dissociation [23], since the isolated $G_{i\alpha}$ subunit is not a good pertussis-toxin substrate [2,15–17]. It has been suggested that the α_i -subunit dissociated from $\beta\gamma$ may be released to the cell cytoplasm [31]. If this occurs, a measurable decrease in immunoreactive $G_{i\alpha}$ in membranes from thrombin-stimulated platelets would be predicted and should be detectable, since thrombin treatment can lead to a decrease of 50% in G_i ADP-ribosylation. To test this hypothesis, an immunoblot analysis was conducted on membrane proteins from thrombin-stimulated platelets (Fig. 1). Thrombin stimulation led to a marked decline in both subsequent pertussis-toxin-dependent ADP-ribosylation (70% in Fig. 1, lower panel, +PT) and the amount of immunoreactive $G_{i\alpha}$ detected in blots (Fig. 1, upper panel, +PT). However, this decline in immunoreactive protein was only apparent with ADP-ribosylated $G_{i\alpha}$. Thrombin had no effect on the amount of immunoreactive $G_{i\alpha}$ detectable in platelet membranes untreated with pertussis toxin (Fig. 1, upper panel, –PT). The results showed that immunoblot detection of $G_{i\alpha}$ is increased when $G_{i\alpha}$ is ADP-ribosylated. This was confirmed by comparing the sensitivity of immunoblot detection of ADP-ribosylated versus unmodified purified bovine brain G_i and G_o (Fig. 2). Pertussis-toxin treatment of purified G_i/G_o resulted in ADP-ribosylation of the α -subunit and concomitant enhanced immunostaining. The cause of this altered sensitivity of immunoblot detection is unknown, but has previously been observed [27].

It is clear that, although thrombin stimulation can inhibit ADP-ribosylation of G_i (Fig. 1, lower panel, +PT), there is no corresponding loss of immunoreactive $G_{i\alpha}$ from platelet membranes (Fig. 1, upper panel, –PT). On Western blots, no immunoreactive $G_{i\alpha}$ was detected in the cytosolic fractions from thrombin-stimulated platelets (results not shown), even though cytosolic $G_{i\alpha}$ would have been detectable if released from membranes in proportion to the level of ADP-ribosylation. The data do not preclude the possibility of minor amounts of $G_{i\alpha}$ being released from membranes in response to thrombin stimulation. Therefore we conclude that, although thrombin can trigger a dissociation of the α subunit of G_i , resulting in decreased ADP-ribosylation, there is no evidence *in vivo* to suggest that the α -subunit is released from the platelet membrane.

Characterization of a soluble $G_{i\alpha}$ protein

While conducting a search for immunoreactive $G_{i\alpha}$ in platelet cytosol, we discovered a 40 kDa soluble protein which is ADP-

ribosylated in a pertussis-toxin-dependent manner (Fig. 3). The ADP-ribosylation by pertussis toxin of this soluble protein was completely inhibited in thrombin-stimulated platelets (Fig. 3). Thrombin is the only agonist which rapidly and significantly inhibited the pertussis-toxin-dependent ADP-ribosylation of both membrane $G_{i\alpha}$ (60% inhibition after 5 s) and the soluble

Fig. 1. Thrombin stimulation causes a decrease in immunoblot-detectable ADP-ribosylated membrane $G_{i\alpha}$

Samples of platelet suspensions were removed 15 and 10 s before (C_1 , C_2) and 5, 15 and 30 s after thrombin (TH) addition (2 units/ml) and were added to portions of an ADP-ribosylation cocktail either lacking (–PT) or containing (+PT) pertussis toxin. Platelet membranes were isolated and subjected to electrophoresis and Western blotting with antiserum AS/7 as described in the Experimental section. The upper panel shows the developed immunoblot and the lower panel an autoradiograph of the same blot. ' $G_{i\alpha}$ ' indicates the electrophoretic mobility of bovine brain $G_{i\alpha}$.

Fig. 2. ADP-ribosylation enhances the immunoblot detection of bovine brain $G_{i\alpha}$

A 7.5 μ g portion of a mixture of purified bovine brain G_i and G_o was added to an ADP-ribosylation cocktail either lacking (–) or containing (+) pertussis toxin (PT). Electrophoresis and Western blotting were conducted as described in the Experimental section. The immunoblot is shown in panel (a) and its autoradiograph in panel (b). Lane 1 contained 7.5 μ g of bovine brain G_i/G_o as standard. The cocktail buffer used in lanes 2–5, 8 and 9 was 20 mM-Hepes (pH 7.4) and was buffer A in lanes 6 and 7. Saponin was absent from the ADP-ribosylation cocktail in lanes 4 and 5. Before electrophoresis, only samples in lanes 8 and 9 were boiled.

Fig. 3. Platelets contain a membrane and a soluble thrombin-responsive G-protein that is ADP-ribosylated in a pertussis-toxin-dependent manner

Samples of platelet suspensions were removed before (C_N , C) and 5 and 15 s after thrombin (TH) addition (1 unit/ml) and were added to portions of an ADP-ribosylation cocktail either lacking (–PT) or containing (+PT) pertussis toxin. The control sample C_N was added to ADP-ribosylation cocktail containing 1 mM unlabelled NAD^+ . Supernatant (panel a) and pellet (panel b) proteins were separated by SDS/PAGE. An autoradiograph of the dried gel is shown. The positions of 42 kDa platelet actin (A) and 41 kDa bovine brain $G_{i\alpha}$ are indicated as molecular-mass markers.

Fig. 4. Effect of various platelet agonists on subsequent pertussis-toxin-dependent ADP-ribosylation of membrane and soluble G-proteins

Samples of platelet suspensions were removed 15 and 10 s before (C_1 , C_2) and 5, 15 and 60 s after agonist addition and were added to portions of an ADP-ribosylation cocktail containing pertussis toxin. Agonists used were: thrombin (TH) at 1.4 unit/ml; vasopressin (VP) at 1.9 μM ; adrenaline (AD) at 100 μM . Supernatant (S) and pellet (P) proteins were separated by SDS/PAGE. An autoradiograph of the dried gel is shown. The arrow points to the 40 kDa soluble ADP-ribosylated protein. The positions of 42 kDa actin (A) and 41 kDa bovine brain $G_{i\alpha}$ are indicated as molecular-mass markers.

$G_{i\alpha}$ protein (80 % inhibition after 5 s) (Fig. 4). Adrenaline and vasopressin were without effect. Also evident in Figs. 3 and 4 is an actin-sized protein which is ^{32}P -labelled in a pertussis-toxin-independent manner in response to thrombin stimulation. This labelling may be due to thrombin-stimulated endogenous ADP-ribosylation, as recently reported by Brüne *et al.* [32] and also observed by us (J. M. Gennity & W. Siess, unpublished work). It is interesting that only thrombin, but not vasopressin or adrenaline, stimulates the ADP-ribosylation of that 42 kDa protein (Fig. 4).

The ADP-ribosylated soluble protein had a slightly higher electrophoretic mobility than the ADP-ribosylated membrane $G_{i\alpha}$ (Fig. 5), indicating an apparent molecular mass (40 kDa) slightly lower than the main $G_{i\alpha}$ protein in platelet membranes ($G_{i\alpha-2}$; 41 kDa). The presence of excess unlabelled NAD^+ inhibited labelling of the soluble protein with [^{32}P]NAD $^+$ (Fig. 3), and labelling could be achieved with [adenosine- ^{14}C]NAD $^+$ (Fig. 5). The covalent modification catalysed by pertussis toxin is therefore an authentic ADP-ribosylation. Like $G_{i\alpha}$, the soluble protein is not a substrate for cholera toxin (results not shown).

Experiments in which the pertussis-toxin-dependent ADP-ribosylation was carried out after separation of platelet supernatant and particulate fractions showed that the presence of platelet membranes was required for the ADP-ribosylation of the soluble $G_{i\alpha}$ protein. In the absence of platelet membranes, ADP-

ribosylation of the soluble $G_{i\alpha}$ protein was not observed (Fig. 6). Interestingly, in these experiments a further soluble ADP-ribosylated protein with higher molecular mass was observed ('X') that was responsive to thrombin. The decrease of ADP-ribosylation of that protein after pretreatment of platelets with thrombin was blocked by prior addition of leupeptin (Fig. 6). The 41 kDa protein in platelet membranes ($G_{i\alpha}$) could be ADP-ribosylated by pertussis toxin in the absence of cytosol (results not shown). Thrombin pretreatment of intact platelets decreased the subsequent ADP-ribosylation of the membrane 41 kDa protein, an effect that was prevented again by the prior addition of leupeptin (results not shown).

Immunoprecipitation studies were performed to characterize further the soluble $G_{i\alpha}$ protein. A specific antiserum against $G_{i\alpha}$ subunits immunoprecipitated the soluble 40 kDa ADP-ribosylated protein (Fig. 7). It also precipitated the membrane 41 kDa ADP-ribosylated protein, as observed previously [11,29]. Both proteins showed a decrease in pertussis-toxin-dependent ADP-ribosylation after pretreatment of platelets with thrombin. The 41 kDa ADP-ribosylated protein present in these experiments to some degree in the soluble fraction is most likely due to a contamination with platelet membranes. Since in these

Fig. 5. Thrombin-responsive soluble G-protein can be ADP-ribosylated with [14 C]NAD $^{+}$ and is smaller than membrane $G_{i\alpha}$

Samples of platelet suspension were removed 15 and 10 s before (C_1 , C_2) and 5, 20 and 60 s after thrombin (TH) addition (1.5 units/ml) and were added to portions of an ADP-ribosylation cocktail containing pertussis toxin and 100 μ M-[adenosine- 14 C]NAD $^{+}$ (600 mCi/mmol) in place of [32 P]NAD $^{+}$. Supernatant (S) and pellet (P) proteins were separated and subjected to electrophoresis and Western blotting with AS/7 as described in the Experimental section. An autoradiograph of the immunoblot is shown. The position of the soluble 40 kDa G protein is indicated at the left. The positions of 42 kDa actin (A) and 41 kDa bovine brain $G_{i\alpha}$ are indicated as molecular-mass markers at the right.

Fig. 6. Pertussis-toxin-dependent ADP-ribosylation of the soluble $G_{i\alpha}$ -protein requires the presence of platelet membranes

Lanes 1–8: samples (80 μ l) of platelet suspensions were removed before (lanes 1, 2, 5, 6) or 10 s (lanes 3, 7) and 30 s (lanes 4, 8) after thrombin (1 unit/ml) addition. Samples in lanes 5–8 contained leupeptin (1 mM) before thrombin addition. Saponin (125 μ g/ml) and leupeptin (1 mM) were then added, and the samples were left for 15 min at 37 $^{\circ}$ C before centrifugation in a microfuge at 15000 g for 5 min. The supernatant was removed and incubated with an ADP-ribosylation cocktail. Lanes 9, 10: samples (80 μ l) of platelet suspensions were removed before (lane 9) or 30 s after (lane 10) thrombin addition, and were transferred to ADP-ribosylation cocktail and incubated for 1 h at 37 $^{\circ}$ C. Then the samples were centrifuged in a microfuge for 5 min to obtain the supernatant. At the end of the ADP-ribosylation, portions (50 μ l) of all samples were transferred into 10% trichloroacetic acid as described in the Experimental section. The proteins were separated by vertical SDS/PAGE (10% gel, 20 cm long). An autoradiograph of the dried gel is shown. The localization of the soluble pertussis-toxin-dependent ADP-ribosylated protein (40 kDa) and the endogenously ADP-ribosylated protein (39 kDa) is shown. See also Fig. 3.

immunoprecipitation experiments the platelet concentration was higher (in order to concentrate the platelet cytosol and to detect better the 40 kDa soluble protein), a contamination of the soluble fraction with membranes seems likely. Specific antisera against $G_{o\alpha}$ and $G_{i\alpha-1}$ proteins did not immunoprecipitate

Fig. 7. Pertussis-toxin-dependent ADP-ribosylated proteins from soluble and particulate platelet fractions are immunoprecipitated by a $G_{i\alpha}$ antiserum

Saponized platelets were [32 P]ADP-ribosylated by pertussis toxin, and proteins from platelet supernatant (lanes 1–4) or particulate (lanes 5–8) fractions were incubated with rabbit antiserum AS/7 (lanes 1, 4, 5, 8; recognizes all G_i subunits), LD/2 (lanes 2, 6; recognizes $G_{i\alpha-1}$) or GO/1 (lanes 3, 7; recognizes $G_{o\alpha}$), and precipitated with BSA-coated Protein A-Sepharose. Platelets in lanes 4 and 8 were pretreated with thrombin (1 unit/ml) for 10 s. An autoradiograph of the dried gel after SDS/PAGE is shown. Positions of the soluble 40 kDa G-protein and 41 kDa membrane $G_{i\alpha}$ are shown. In control experiments using purified brain G_o/G_i , antisera GO/1 and LD/2 precipitated proteins corresponding to G_o and G_i respectively (results not shown).

Fig. 8. Thrombin inhibits pertussis-toxin-dependent ADP-ribosylation of the soluble $G_{i\alpha}$, but not the membrane $G_{i\alpha}$ in leupeptin-treated platelets

Samples of 1 mM-leupeptin-treated platelet suspensions were removed before (C_1 , C_2) and 5 and 15 s after thrombin (TH) addition (1.9 units/ml) and were added to portions of an ADP-ribosylation cocktail containing pertussis toxin. Supernatant (S) and pellet (P) proteins were separated by SDS/PAGE. An autoradiograph of the dried gel is shown. The positions of 42 kDa actin (A) and 41 kDa bovine brain $G_{i\alpha}$ are indicated as molecular-mass markers.

soluble or membrane ADP-ribosylated proteins (Fig. 7). These results indicate that the soluble 40 kDa protein belongs to the $G_{i\alpha}$ protein family, but is most likely not $G_{i\alpha-1}$.

Thrombin inhibits the pertussis-toxin-dependent ADP-ribosylation of the soluble $G_{i\alpha}$ protein

It was possible to demonstrate functional differences in the soluble 40 kDa and the membrane 41 kDa $G_{i\alpha}$ proteins. The proteinase inhibitor leupeptin is capable of preventing thrombin-stimulated platelet activation [33,34]. We observed that leupeptin inhibited thrombin-induced platelet aggregation (results not shown) and in parallel prevented thrombin-induced inhibition of membrane $G_{i\alpha}$ ADP-ribosylation (Fig. 8). In contrast, leupeptin did not affect the thrombin-induced inhibition of ADP-ribosylation of the soluble 40 kDa protein (Fig. 8).

Furthermore, mild chymotrypsin pretreatment of platelets [19,20] was found to abolish completely the inhibition by thrombin of membrane $G_{i\alpha}$ ADP-ribosylation. In contrast, chymotrypsin digestion inhibited by only 30% the thrombin-induced decrease in ADP-ribosylation of the soluble protein

Fig. 9. Thrombin inhibits pertussis-toxin-dependent ADP-ribosylation of the soluble $G_{i\alpha}$, but not the membrane $G_{i\alpha}$ in chymotrypsin-treated platelets

Intact platelets were incubated without (–CHY) or with chymotrypsin (+CHY) as described in the Experimental section. Samples of platelet suspensions were removed before (C) and 5 and 15 s after thrombin (TH) addition (1 unit/ml) and were added to portions of an ADP-ribosylation cocktail containing pertussis toxin. Supernatant (S) and pellet (P) proteins were separated by SDS/PAGE. An autoradiograph of the dried gel is shown. The position of the soluble 40 kDa G-protein is indicated at the right. The positions of 42 kDa actin (A) and 41 kDa bovine brain $G_{i\alpha}$ are indicated as molecular-mass markers at the left.

Fig. 10. Effect of various concentrations of thrombin on the subsequent pertussis-toxin-dependent ADP-ribosylation of membrane $G_{i\alpha}$ and soluble $G_{i\alpha}$

Samples of platelet suspensions were removed 5 s after addition of thrombin at the indicated concentrations and were added to portions of an ADP-ribosylation cocktail containing pertussis toxin. Supernatant (S) and pellet (P) proteins were separated by SDS/PAGE, and the radioactivity in gel slices containing membrane $G_{i\alpha}$ (●) and the soluble $G_{i\alpha}$ protein (○) was determined.

(Fig. 9). The quantity of chymotrypsin used in this experiment did not cause any noticeable proteolysis of membrane or cytosolic proteins, as judged by examination of Coomassie-Blue-stained SDS/polyacrylamide gels, and did not prevent platelet aggregation (results not shown). Platelet pretreatment with 50-fold greater chymotrypsin concentrations blocked the response to thrombin of both the soluble and the membrane $G_{i\alpha}$, extensively proteolysed membrane proteins, and prevented aggregation (results not shown).

There is no major difference between the membrane and the soluble $G_{i\alpha}$ protein in their response to different doses of thrombin. ADP-ribosylation of both proteins is inhibited by 50% at approx. 0.2–0.3 unit of thrombin/ml (Fig. 10). This suggests that both proteins are coupled to the same thrombin receptor, although the possibility cannot be eliminated that both

proteins are individually coupled to different receptors with a similar affinity for thrombin.

DISCUSSION

The observation that thrombin stimulation can inhibit the subsequent pertussis-toxin-dependent ADP-ribosylation of $G_{i\alpha}$ is believed to indicate that receptor activation results in the dissociation of the α -subunit from $\beta\gamma$ of G_i [23,24]. This subunit dissociation could also be accompanied by a movement of $G_{i\alpha}$ from plasma membrane to the cytosol [31]. We have been unable to observe any agonist-stimulated decrease in immunodetectable $G_{i\alpha}$ associated with platelet membranes which would quantitatively correspond to the 70% inhibition of ADP-ribosylation caused by thrombin. Our immunoblot analysis would have been able to detect cytosolic $G_{i\alpha}$ if the quantity released from the membrane was proportional to ADP-ribosylation. Our data do not eliminate the possibility that minor amounts of $G_{i\alpha}$ are solubilized in response to thrombin stimulation. Therefore our results suggest that $G_{i\alpha}$, although dissociated from β and γ subunits, is not released from the plasma membrane of activated platelets. Instead, we detected a soluble protein, ADP-ribosylated in a pertussis-toxin-dependent manner, that responds specifically to thrombin stimulation of platelets. This protein is slightly smaller than membrane $G_{i\alpha}$ in molecular mass. It is present in platelets at only 5% the level of $G_{i\alpha}$, assuming both proteins are ADP-ribosylated to equivalent extents. Owing to the small amounts of soluble $G_{i\alpha}$ proteins, we have been unable to detect on immunoblots any soluble proteins which react with anti- $G_{i\alpha}$ antisera. However, the ADP-ribosylated soluble 40 kDa protein could be immunoprecipitated with antisera against $G_{i\alpha}$ but not against $G_{i\alpha-1}$.

It has been demonstrated that the pertussis-toxin substrates in platelet membranes are $G_{i\alpha-2}$ and $G_{i\alpha-3}$ (apparent molecular mass 40–41 kDa), with $G_{i\alpha-2}$ being much more abundant than $G_{i\alpha-3}$ [10–13]. $G_{i\alpha-1}$ is absent from [12], or only present in minute amounts [13] in, platelets, whereas $G_{o\alpha}$ could not be detected [11–13], results that were partly confirmed by our study. $G_{i\alpha}$ proteins are tethered to the plasma membrane via their N -terminal end [35]. Myristoylation of glycine in position 2 of $G_{i\alpha}$ is essential for its membrane attachment [36–38]. The soluble $G_{i\alpha}$ protein found in our study in platelets might not be myristoylated; it could be lacking the N -terminal glycine carrying the myristoylation site or be a clipped product of the major ADP-ribosylated membrane $G_{i\alpha}$. This also could explain its faster mobility on SDS/PAGE. As the C -terminus is the site of ADP-ribosylation, and G-protein ADP-ribosylation requires an interaction of the N -terminus of the α -subunit with the β -subunit, the

Table 1. Characteristics of the α_i subunits of two G-proteins coupled to the platelet thrombin receptor

Characteristic	α_i subunit	
	Membrane	Soluble
Apparent molecular mass after ADP-ribosylation	41 kDa	40 kDa
Abundance	Major	Minor
Pertussis-toxin substrate	Yes	Yes
Cholera-toxin substrate	No	No
Receptor coupling	Thrombin receptor	Thrombin receptor
Chymotrypsin*	Blocks	None
Leupeptin*	Blocks	None

* Effect of the indicated treatment on thrombin-induced inhibition of pertussis-toxin-dependent ADP-ribosylation.

cytosolic G-protein must retain considerable essential structure. In effect, the presence of this soluble G-protein in resting platelets and retention of coupling to the thrombin receptor are evidence of a distinct biological function.

Although a membrane and a soluble G_iα protein appear to be coupled to the thrombin receptor, they can be functionally differentiated. Leupeptin or chymotrypsin treatment of platelets prevented the thrombin-stimulated inhibition of membrane G_iα ADP-ribosylation, but did not block the inhibition of ADP-ribosylation of the soluble protein. Characteristics of the two proteins are summarized in Table 1. Although the structural relationship of the two proteins remains to be clearly established, the differences between the membrane and the soluble G_iα protein suggest that the latter is the α_i subunit of a G-protein which is functionally distinct from membrane G_iα. Since ADP-ribosylation of the soluble G_iα protein was only observed in the presence of platelet membranes, we conclude that the soluble G_iα protein was ADP-ribosylated by pertussis toxin while bound to putative β- and γ-subunits attached to the plasma membrane. This ADP-ribosylated subunit could then dissociate from the membrane to account for the observed supernatant subcellular location in saponized platelets. Occupancy of the thrombin receptor could lead to a conformational or covalent change of the soluble G_iα and hence to its lack of interaction with membrane βγ-subunits that is required for ADP-ribosylation by pertussis toxin.

Minor amounts of a pertussis-toxin-sensitive soluble G-protein having the same molecular mass as the main pertussis-toxin substrates (40 kDa) in membranes have been previously observed in bovine and human neutrophils [39–41]. The quantity of cytosolic pertussis-toxin-sensitive α_i-2 in renal epithelial cells remained unaltered, whereas the amount of membrane-associated protein increased upon cell stimulation [42]. The functional and biological relevance of these soluble proteins remains unclear. Mutations which prevent myristoylation of α_i expressed in COS cells interfered with membrane association [37,38], but did not prevent pertussis-toxin-dependent ADP-ribosylation [38]. This indicates that soluble α-subunits are still capable of interaction with βγ-subunits and is consistent with the present identification of a functional soluble G-protein in platelets. A soluble pertussis-toxin substrate having the same molecular mass as membrane G_iα (41 kDa) has previously been described in platelets [43]. It was speculated that this soluble G-protein may be responsible for the GTP-dependent activation of purified soluble phospholipase C. This fits well with our speculation that the soluble G-protein may be a regulatory protein which couples the thrombin receptor to a chymotrypsin-insensitive response (see the discussion below). Another cytosolic platelet G-protein has been observed [44], but, on the basis of its different size and toxin-sensitivity, is distinct from the protein described here.

The platelet thrombin receptor(s) has not yet been identified. Thrombin has hormone-like and enzyme-like properties, and it interacts with the platelet surface at several specific sites (for references see [18]). The proteinase inhibitor leupeptin prevents thrombin-catalysed proteolysis and platelet activation, without inhibiting thrombin binding [45]. Though the exact mode of action is not known, mild chymotrypsin treatment of intact platelets apparently cleaves a thrombin-sensitive ligand, thereby inhibiting certain thrombin-stimulated responses [19,20]. The results of our experiments with leupeptin and chymotrypsin treatment of platelets indicate that thrombin-receptor occupancy alone is sufficient to trigger the inhibition of the pertussis-toxin-dependent ADP-ribosylation of the soluble G_i protein. Activation of that G_i protein might be an initial event after binding of thrombin to its receptor, but alone it is insufficient to stimulate

platelet activation. In contrast, both receptor occupancy and thrombin-induced proteolysis are required for the inhibition of membrane G_iα ADP-ribosylation. This interpretation fits well the findings of Detwiler and co-workers [19]. They found that chymotrypsin treatment of intact platelets blocked thrombin-stimulated responses (adenylate cyclase regulation and arachidonate release) which are recognized as being coupled to G_i, whereas responses such as activation of phospholipase C and protein kinase C (chymotrypsin-insensitive responses), which are coupled to as yet undefined regulatory proteins, were only decreased. To explain these findings, the proposal was made that either two thrombin receptors or one receptor coupled to two variant signal-transducing mechanisms are present in platelets. Under similar conditions (chymotrypsin or leupeptin treatment) we observe a selective inhibition of membrane G_i activation with retention of thrombin-stimulated soluble G_i protein activation. This suggests that two distinct platelet G-proteins are activated by thrombin. The similarity in the dose-response curves for thrombin activation of these two G-proteins favours the hypothesis that both effectors are coupled to one thrombin receptor. It will be important to determine whether the thrombin-coupled 'soluble' G_iα protein described here is a regulatory protein coupled to any of the chymotrypsin-insensitive responses described by Detwiler [19,20].

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