The thrombin receptor in human platelets is coupled to a GTP binding protein of the $G\alpha_q$ family

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Abstract The thrombin receptor is a G protein-coupled receptor, but the G proteins functionally coupled to this receptor in human platelets are not yet definitively identified. Thrombin stimulation of platelets leads to phospholipase C-mediated increases in intracellular calcium, and previous studies have suggested that the thrombin receptor is coupled to members of the G_q family. We now demonstrate direct GTPase activation by thrombin receptor activation peptide (TRAP) in human platelet membranes, and specific inhibition of TRAP-activated GTPase by antibodies to G_q. These data demonstrate functional coupling of the thrombin receptor to a member of the G_q family.

Key words: G protein; Guanosine triphosphate; Cell surface receptor; Blood platelet; Signal transduction; Second messenger system

1. Introduction

The serine protease thrombin has an important role in hemostasis and thrombosis, inflammation and cell proliferation [1]. Platelet stimulation by thrombin occurs by activation of a recently cloned member of the seven transmembrane receptor family via a novel proteolytic mechanism [2,3]. Thrombin stimulation of platelets leads to increases in phospholipase C (PLC) activity, phosphoinositide metabolism, cytosolic calcium levels, protein phosphorylation, and eicosanoid formation, each of which appears to be mediated via heterotrimeric GTP binding protein(s) (G proteins) coupled to the receptor [4,5]. Platelets contain at least nine G_α forms, but the identity of G protein(s) coupled to the thrombin receptor and phospholipase C (PLC) activation in platelets is controversial. Pertussis toxin inhibits thrombin-mediated phosphoinositide metabolism in permeabilized platelets [6], raising the possibility that members of the G_i family can partially mediate PLC activation by thrombin. However, Grandt and co-workers found that approximately 70% of platelet G protein activation by thrombin is pertussis toxin-insensitive [7] and Houslay et al. similarly found a significant pertussis-insensitive GTPase coupled to thrombin stimulation [8]. Studies in several different cells have shown the identity of 'G_p', the pertussis-insensitive, PLC-coupled GTP binding protein, to be a member of the G_q family [9–11]. Platelets are known to contain G_q, which co-purifies with the thromboxane receptor from these cells [12] and recently has been demonstrated to be coupled functionally to this receptor using inhibitory antibodies [13]. Using a heterologous expression system, Hung and colleagues demonstrated the cloned thrombin receptor couples to at least two distinct G proteins, found the G protein coupled to phosphoinositide hydrolysis to be pertussis toxin-insensitive, and suggested this G protein was a member of the G_q family [14]. We therefore sought to demonstrate functional coupling of G_q to the thrombin receptor, using platelet membranes to study the activation of the GTPase by thrombin and antibodies to G_α subunits to investigate the role of G_q and other G proteins in thrombin-activated GTPase. The data show (i) TRAP potently activates platelet membrane GTPase activity in a dose-dependent manner, (ii) this GTPase activity can be inhibited by anti-G_q antibodies, and (iii) this inhibition by anti-G_q is prevented by neutralization of the inhibitory antibodies with their cognate peptide.

2. Experimental

2.1. Materials

[γ-^{32}P]GTP was a kind gift from Amersham International (5,000 Ci/mmol; 10 μCi/μl). EGTA, $MgCl_2$, dithiothreitol, ATP and phosphocreatinine were from Sigma (St. Louis, MO). Creatine phosphokinase was from Calbiochem (San Diego, CA). U46619, the stable analogue of thromboxane A_2, was from Cayman Chemical Co. (Ann Arbor, MI). Thrombin receptor agonist peptide (TRAP), amino acid sequence SFLLRNPNDKYGPF [15], was generously donated by Calbiochem.

2.2. Platelet membrane preparations

Platelet membranes were prepared exactly as described [13] from just-outdated platelet concentrates obtained from a hospital blood bank. Aliquots were stored at −80°C until the day of each assay, at which time they were thawed, diluted in 30 vols. of ice-cold 10 mM triethanolamine HCL, 5 mM EDTA, pH 7.4, and centrifuged at 39,000 × g for 20 min. Pellets were resuspended and washed twice more

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in the same manner, and then resuspended finally in ice-cold 10 mM triethanolamine-Cl, 100 nM NaCl, pH 7.4, for the assay. Protein concentrations were determined daily using the Bradford method (Bio-Rad), with BSA as a standard. Final membrane solutions used in each assay were consistently in the range of 0.45–0.55 mg/ml.

2.3. GTPase assay

All experiments were done between July 4 and July 24, 1994, by students and faculty members of the 1994 MBL Physiology Course, working in pairs. All assay points were performed in triplicate unless a pipeting error was noted in writing before scintillation counting of the GTPase assay, in which case duplicates were used (less than 1% of total assay points). The high affinity GTPase in human platelet membranes was measured by a modification of the method of Aktories and Jakob [16], essentially as reported by Shenker et al. [13]. Reactions were conducted in a final volume of 100 μl, and were initiated by the addition of 20–45 μg of platelet membranes in a volume of 50 μl to tubes containing reaction mixture without or with specific agonists. The final reaction mixture contained 0.4 μM GTP, including 0.6–0.9 μCi of [γ-^{32}P]GTP ($1.5–2 \times 10^6$ dpm), as well as 100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ATP, 5 mM phosphocreatine, 100 U/ml creatine phosphokinase, 0.2% bovine serum albumin, 50 mM triethanolamine-HCl, pH 7.4, and indicated concentrations of agonists (U46619 or TRAP). Reactions were terminated with 5% activated charcoal/20 mM phosphoric acid, and high affinity GTPase activity was calculated exactly as described [13] and expressed as pmol ^{32}P released per min per mg of platelet protein. Antibody pretreatments were performed by combining 3–5 μl of antibody solution with membrane suspensions for 60–90 min at 25°C, with gentle agitation. For the peptide experiments, antibodies were combined with their cognate peptides in microcentrifuge tubes for 30–45 min at 25°C, at which time the membranes were added to the tubes for a further 60–90 min at the same temperature. Statistical comparisons were made by two-tailed Student's *t*-test with unequal variance, using Microsoft Excel.

2.4. Antibodies, peptide antigens and membrane pretreatments

Commercially available antibodies to the various G_α subunits were used in initial experiments (see Table). Commercially available anti-G_α antibodies used included; (G protein sequence used as peptide antigen; stock concentration): from Calbiochem: G_{i3}/G_o (AA345–354; 1:20,000); G_s (AA385–394; 1:24,000); and from Dupont-NEN (G-protein sequence used as peptide antigen): QL [anti-G_q, G_{11}] (QLNLKEYNLV). For experiments with commercial antibodies, stocks were diluted 1:133–1:350 directly into the platelet membrane suspension.

After evidence of antibody inhibition was obtained with these antisera, affinity purified antibodies were used to confirm the specificity of observed inhibition (made and generously provided by P. Goldsmith, A. Spiegel and colleagues). All affinity purified antisera were against the C-terminal decapeptide of specific G_α subunits and have been extensively characterized [13,17]. These include: QL, raised against $G_{q/11}$ (peptide antigen = QLNLKEYNLV); AS, raised against $G_{i1/i2}$ (peptide antigen = KENLKDCGLF); and QN, raised against G_z (peptide antigen = QNNLKYIGLC). Affinity-purified antibodies were used at a final concentration of 10 μg/ml in all cases. Peptides were synthesized and HPLC-purified as described [13,17] and stock solutions (2.5–10 mg/ml) were diluted as described in section 3.

3. Results and discussion

Initial experiments defined the platelet membrane GTPase dose–response to the thrombin receptor activation peptide, as well as to the thromboxane analog U46619 (Fig. 1). Resting (unstimulated) platelet membranes showed a basal GTPase activity of 9.88 ± 0.57 pmol/min/mg platelet protein (mean ± S.E.M., $n = 25$), consistent with previous reports of basal GTPase activity in freshly prepared platelet membranes [13,16]. Stimulation of membranes with the thromboxane analog U46619 or TRAP led to a specific, dose-dependent increase in GTPase activity by each agonist. U46619 stimulated the hydrolysis of GTP with an EC_{50} of 4 μM and a maximal increase in GTPase activity of 4.51 pmol/min/mg platelet protein (from 9.88 pmol/min/mg to 14.39 pmol/min/mg at 100 μM).

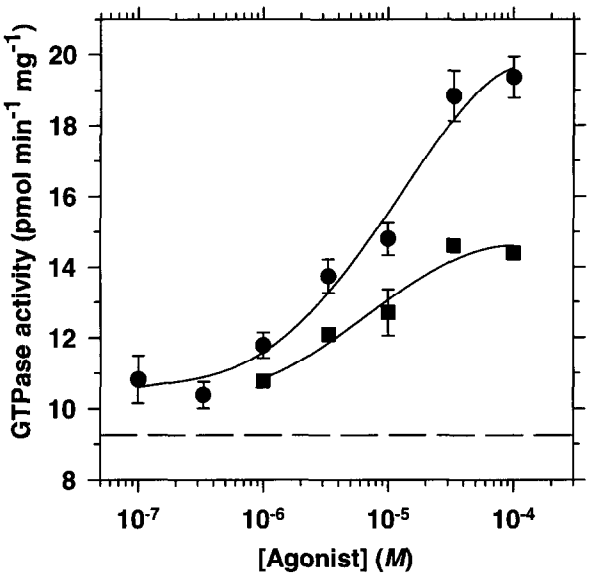

Fig. 1. Dose–response curves for the activation of platelet membrane GTPase activity by TRAP (●) and the thromboxane A2 analogue U46619 (■). Stimulation of platelet GTPase activity by the thromboxane analogue U46619 led to a specific, dose-dependent increase in GTPase activity, with an EC_{50} of 4 μM. Stimulation of membranes with TRAP also led to a specific, dose-dependent increase in GTPase, with an EC_{50} of 6 μM. Basal GTPase activity in the absence of agonist was 9.88 pmol/min/mg protein (dotted line; $n = 25$).

TRAP also stimulated a specific, dose-dependent increase in GTPase activity. The EC_{50} for TRAP-stimulated GTPase was 5 μM. Direct measurement of TRAP-stimulated GTPase activity in platelets has not been reported previously, but this EC_{50} is similar to those reported for TRAP stimulation of platelet aggregation (1.3–7 μM) [18,19]. Interestingly, the net maximal increase in TRAP-specific GTPase activity observed was 8.41 ± 1.07 pmol/min/mg platelet protein (from 9.88 to 18.29 ± 1.51 at 100 μM TRAP), approximately twice that observed for U46619 stimulation. This suggests that the membranes have more thrombin receptors than thromboxane receptors, that the thrombin receptor couples more efficiently to the same number of G protein(s), or that the thrombin receptor is coupled to a greater number of G proteins than the thromboxane receptor.

Antisera against the C-terminal decapeptide of G_α subunits have proven to be useful as probes for the coupling of receptors with their G proteins [13,17,20]. A series of experiments was therefore performed in an attempt to inhibit agonist-stimulated GTPase activity by treatment of the platelet membranes with antibodies raised to the carboxyl-termini of G_α subunits. Such antibodies have previously been shown to inhibit platelet membrane GTPase activity to specific agonists [13,17]. Initial experiments evaluated TRAP or U46619-stimulated GTPase activity in the absence or presence of membrane pretreatment with several commercially available inhibitory C-terminal antibodies (Table 1). Based on the dose–response curves of Fig. 1, antibody inhibition of the GTPase stimulated by 10 μM of the respective agonist was tested. The thromboxane analogue U46619-stimulated GTPase activity was significantly inhibited

by anti-G_q antibody QL ($P < 0.0001$). We also found lesser but significant inhibition of the U46619-stimulated GTPase by G_s antibody in this experiment, but no effect of the C-terminal antibodies directed against G_{i3}/G_o. In the experiments with TRAP, significant inhibition of stimulated GTPase activity was observed only with the anti-G_q antibody QL. These experiments demonstrated an inhibitory effect of the anti-G_q antibody, confirmed the coupling of G_q to the thromboxane receptor reported previously [13], and raised the possibility that G_q was functionally coupled to the thrombin receptor in human platelet membranes. We sought next to determine the specificity of the inhibitory effect. To do so, we turned to experiments with affinity purified antibodies. As controls, we used antibodies preabsorbed with the peptides to which they were raised.

The three affinity purified C-terminal G protein antibodies were studied next. These antibodies QL, AS, and QN, to $G_{q/11}$, G_{i1+2} and G_z, respectively, have been previously well characterized [13,17]. In addition, the QL antibody to G_q used is an affinity-purified version of the QL antibody used in the experiments in Table 1. In experiments with the affinity-purified QN and AS at a final concentration of 10 μg/ml, specific inhibition of TRAP-stimulated platelet GTPase activity was not demonstrable (data not shown). In many experiments the antibodies QN and AS did produce some non-specific GTPase inhibition, which was independent of antibody concentration and unaffected by preabsorption of antibody with peptide. Only antibody QL produced marked inhibition of agonist-stimulated GTPase that could be specifically reversed by preabsorption of the antibody with the immunizing peptide (Fig. 2).

In the experiments with affinity purified QL, a small but significant effect of the antibody alone on basal membrane GTPase was noted, consistent with a contribution of the G_q family to basal platelet membrane GTPase activity. This basal effect was reversed by pretreatment of the antibody with its corresponding peptide. At both TRAP concentrations tested, marked and significant inhibition of the agonist-stimulated GTPase by QL was observed (52% inhibition at 10^{-4}M TRAP, 46% inhibition at 10^{-5}M TRAP). In five such experiments, the maximal inhibition of the TRAP-stimulated GTPase was 64.4 ± 9.4%. This partial inhibition of the platelet thrombin-stimulated GTPase by G_q antibody is consistent with the studies of Grandt and colleagues, who found 70% of the platelet thrombin-stimulated GTPase to be pertussis-insensitive [7], and with those of Hung et al., who found thrombin receptor-mediated phosphoinositide hydrolysis occurred via a pertussis toxin-insensitive G protein [14]. Pretreatment of the QL solution with 10 μg/ml of the peptide used to raise the antibody caused reversal of the antibody inhibition of TRAP-stimulated GTPase at both concentrations of TRAP tested.

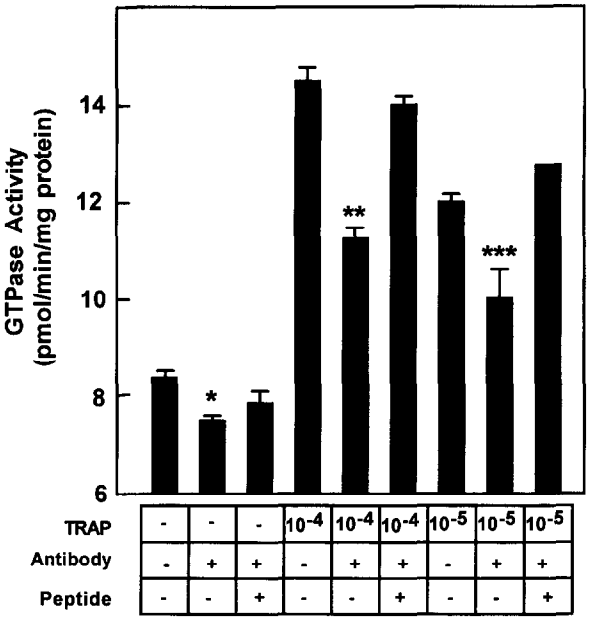

Fig. 2. Specific inhibition of TRAP-stimulated platelet GTPase activity by anti-G_q antibody. Basal (first three bars) and TRAP-stimulated GTPase activity in the absence or presence of preincubation with anti-G_q antibody QL is shown. QL antibody had a small but significant inhibitory effect on basal GTPase activity (*$P = 0.03$), while peptide-preabsorbed QL did not. The QL antibody markedly inhibited TRAP-stimulated GTPase activity at both 10^{-4} and 10^{-5} M TRAP (**$P = 0.0007$; ***$P = 0.04$). This inhibition was reversed by preincubation of the antibody solution with cognate peptide in each case. One of three similar experiments is shown.

Table 1
Platelet membrane GTPase activity after TRAP or U46619 stimulation in the absence or presence of anti-GTP binding protein antibodies

Membrane preincubation	TRAP (10^{-5} M) stimulation of GTPase (Net)	U46619 (10^{-5} M) stimulation of GTPase (Net)
None (control)	5.29 ± 0.256	4.43 ± 0.093
G_{i3}, G_o	4.57 ± 0.536	5.47 ± 2.90
G_s	3.97 ± 0.518	3.27** ± 0.244
QL (G_q)	2.97* ± 0.718	1.03*** ± 0.138

All activities are expressed as pmol/min/mg platelet protein. Basal activity was 8.73 ± 0.939 pmol/min/mg. One of three similar experiments is shown. In all such experiments, 26–27.5 μg of human platelet membrane were incubated with buffer alone or anti-G_α subunit antibodies, as explained in section 2. Two-tailed Student's t-test, with unequal variance: *$P = 0.013$ vs. control, **$P = 0.004$ vs. control, ***$P < 0.0001$ vs. control. All other values are not significant vs. control.

The data presented demonstrate that thrombin receptor activation peptide activates platelet membrane GTPase activity in a dose-dependent manner, which can be inhibited by the anti-G_q antibody QL in a specific fashion. These data are consistent with functional coupling of the human platelet thrombin receptor to one or more members of the G_q family. However, the data do not exclude coupling of the thrombin receptor to other G_α subunits since at the 10 μg/ml concentration of antibody QL tested, on average 36% stimulation of GTPase activity by TRAP was still observed. Significantly higher concentrations of antibody have been used in similar studies previously [13,17] and might very well demonstrate specific inhibition by other G_α antibodies. In fact, based on previous studies, we would predict some of the residual TRAP GTPase is due to coupling to G_i [6–8,14], though this remains to be demonstrated. A recent study by Offermanns and colleagues used photolabeling techniques to demonstrate thrombin receptor association with both G_q and G_{12} family members in platelet membranes [21]. In CCL39 fibroblasts, the thrombin receptor recently has been found to stimulate calcium mobilization and DNA synthesis by members of the G_q and G_o families [22]. Hung and colleagues found phosphoinositide metabolism coupled to a pertussis toxin-insensitive G protein in a heterologous expression system, suggesting the involvement of G_q [14]. However, the data here

are the first to demonstrate direct coupling of the thrombin receptor to G_q GTPase activity in any cell. It is not yet clear which member(s) of the G_q family are involved, especially since the presence of G_{11} in platelets is controversial [5,23]. It next will be important to determine which G proteins other than those of the G_q family are functionally coupled to the thrombin receptor. It will also be important to understand whether individual thrombin receptors routinely activate several different G proteins, and/or if specific thrombin receptor subtypes exist that couple to distinct G proteins.

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