Effects of thrombin, phorbol myristate acetate and prostaglandin D_2 on 40–41 kDa protein that is ADP ribosylated by pertussis toxin in platelets

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Intact platelets were stimulated with thrombin and the amount of GTP-binding protein (G-protein) oligomers was assessed by measuring ADP ribosylation of 40–41 kDa protein by pertussis toxin in isolated membranes. The toxin substrate fell by 57–62% in 10–60 s, but then returned towards normal over 5 min. Recovery was greatly enhanced by removal of thrombin from receptors with hirudin. Phorbol myristate acetate increased ADP-ribosylatable protein, but only back to initial levels prior to PMA. In contrast prostaglandin D_2 plus theophylline (which increase cyclic AMP) did not increase ADP ribosylation, but could completely block the fall of the toxin substrate caused by thrombin. These results indicate that activation of thrombin receptors promotes the dissociation of G-protein oligomers to release free α-subunits, and this effect can be modulated by protein kinase C and cyclic AMP-dependent protein kinase. The possible relationships of these findings to the regulation of stimulus-response coupling in platelets is discussed.

Thrombin *Phorbol ester* *Prostaglandin D_2* *Pertussis toxin*

1. INTRODUCTION

The catalytic activity of adenylate cyclase is regulated by the relative states of dissociation of the guanine nucleotide-binding proteins (G-proteins) G_s and G_i, which are under the control of hormones or agonists and their receptors [1]. Certain G-proteins are now also believed to be involved in stimulus-response coupling by regulating the activity of the specific phosphodiesterase that hydrolyzes PIP_2 to form diacylglycerol and the Ca^{2+}-mobilizing agent IP_3 [2–6]. Pertussis toxin, which causes ADP ribosylation of the α (nucleotide-binding) subunit of intact G_i-$\alpha\beta\gamma$ oligomers, prevents the dissociation of G_i subunits from the oligomer [7], and blocks inhibition of adenylate cyclase by receptors linked to G_i. The toxin also inhibits agonist-induced PIP_2 breakdown and calcium mobilization in neutrophils [8–10], basophilic leukemia cells [11], adipocytes [12], and mast cells [13]. However, stimulation of other cell types by hormones and agonists, although GTP-dependent, is not inhibited by pertussis toxin [6,14,15], and therefore may involve G-proteins which like G_o are less susceptible to the toxin [16].

Thrombin causes breakdown of PIP_2 in platelets with resultant formation of IP_3 [17] and diacylglycerol [18], and mobilization of calcium [19]. Adenylate cyclase activity is also inhibited. Only the latter effect has been shown to be mediated by receptors linked to G_i, which can be inhibited by pertussis toxin in membrane fractions [20]. Intact platelets are not susceptible to pertussis

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toxin, presumably owing to lack of surface receptors necessary for its internalization [21]. However, in permeabilized platelets GTP potentiates formation of diacylglycerol and the secretion caused by calcium and thrombin [22,23], indicating that G-proteins may play a role in stimulus-response coupling in platelets apart from regulation of adenylate cyclase.

To investigate the state of association of G-protein oligomers in intact platelets we assayed the amount of ADP-ribosylatable protein in membranes isolated from platelets that were stimulated by the agonist. Pertussis toxin ADP ribosylates the 41 kDa α-subunit of the intact G_i-$\alpha\beta\gamma$ oligomer, and not free α (with or without bound nucleotide) [24–26]. By this approach we obtained the first direct evidence that thrombin causes a reversible dissociation of G-protein oligomers in intact platelets. Furthermore, this effect was blocked by PGD_2, which increases cyclic AMP. We also present evidence that the state of dissociation/association of the pertussis toxin substrate is also modulated by protein kinase C.

2. MATERIALS AND METHODS

2.1. *Preparation of washed platelets and treatment with thrombin, PGD_2, and PMA*

Human platelets from the Connecticut Red Cross Blood Center were washed as described [20], resuspended in 70 ml of 145 mM NaCl, 5 mM KCl, 10 mM Hepes (pH 7.4), 0.2 mM EGTA, 5.5 mM dextrose, and then pelleted at 750 × *g* (10 min). The platelets were resuspended to 5×10^9 cells/ml in 10 mM Hepes (pH 7.4)-saline (as above) containing 2 mM EGTA and 1 mM $MgCl_2$, and 1.0–2.0 ml aliquots were incubated at 37°C for 10 min. Thrombin, and/or other agents, were added to the platelets and the incubations continued for the prescribed times, until terminated by the addition of 5.0 ml ice-cold medium containing 1 U/ml of hirudin and immediately placed in an ice bath. The platelet membrane fraction was isolated at 0–4°C as described below.

2.2. *Preparation of platelet membranes and ADP ribosylation*

Platelets were pelleted at 750 × *g* (Sorvall RC 5B centrifuge) and resuspended in 30 ml of hypotonic medium: 1 mM $KHCO_3$ (pH 8.3), 1 mM EGTA, 10 μM pepstatin, 10 μM leupeptin and 0.5 mM diisopropylfluorophosphate, and stored on ice for 30–45 min. The swollen platelets were centrifuged at 7700 × *g* (10 min), resuspended in 6 ml hypotonic medium, and homogenized by 20 strokes in a stainless steel Dounce homogenizer with a tight fitting pestle. Unbroken cells were removed by centrifugation at 3000 × *g* (10 min) and the supernatants were diluted to 40 ml in ADP-ribosylation medium: 0.1 M sucrose, 0.67 mM EDTA, 1 mM ATP, 2.5 mM $MgCl_2$, 10 mM thymidine, 0.5 mM DFP, 12 μM [^{32}P]NAD (2 Ci/mol), 1.3 mM DTT, 13.3 mM potassium phosphate and 5 mM Tris-HCl, final pH 7.2. Membranes were obtained by centrifugation at 39000 × *g* for 30 min, then washed and resuspended to 1–3 mg protein/ml in ADP-ribosylation medium. The reaction was started by adding 17 μg/ml of pertussis toxin preactivated with 10 mM DTT at 30°C for 10 min, and terminated after 20 min by adding (to 50 μl of membrane suspension) 25 μl of stopping-solution: 9% SDS, 15% glycerol, 0.05% bromophenol blue, 6% β-mercaptoethanol and 86 mM Tris-HCl, pH 6.7. The samples were heated in a boiling water bath and the proteins were separated [27] on 10.5% polyacrylamide gels with 0.1% SDS. The gels were dried and exposed to Kodak XAR-5 film overnight. The amount of radioactive 40–41 kDa protein was determined by densitometry of the X-ray film.

3. RESULTS AND DISCUSSION

Platelets from 10 donors were exposed to thrombin at concentrations of 0.5, 1.0 and 2.0 units/ml (0.1–0.4 U/10^9 platelets) for 30 s at 37°C and the amount of ADP-ribosylatable protein was measured in the membrane fraction after gel electrophoresis (fig.1A). Stimulation by thrombin caused the amount of [^{32}P]ADP-ribosylated G-protein to fall to only 38% to 43% of the control level (table 1). Since the α-subunit of G_i oligomers, but not free α-subunit, is a substrate for ADP ribosylation these results probably indicate that dissociation of G-protein oligomers occurred. The time course of this effect at two different concentrations of thrombin is shown in fig.2. At 2.0 U/ml, thrombin reduced [^{32}P]G-protein by 73% within 10 s, thereafter it slowly increased to

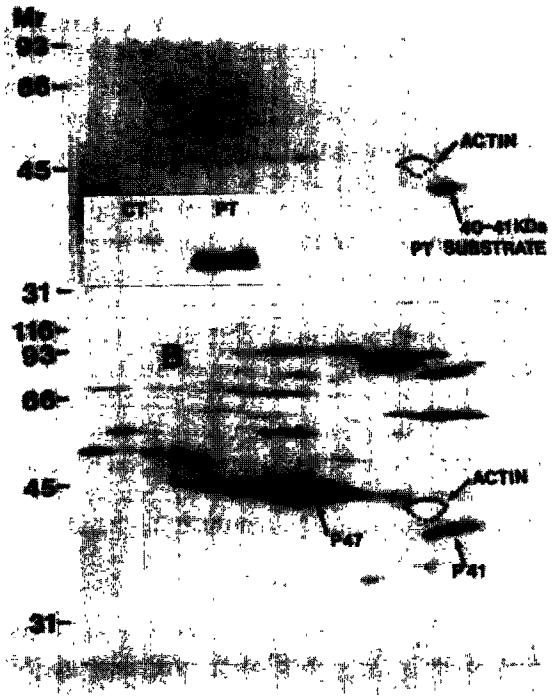

Fig.1. (A) Two-dimensional gel electrophoresis of membranes from unstimulated platelets showing position of [^{32}P]ADP-ribosylated 40–41 kDa protein (pertussis toxin substrate). Inset shows SDS-gel of 45 kDa and 40–41 kDa proteins ADP ribosylated by cholera toxin (CT) and pertussis toxin (PT), respectively (molecular mass scale is not same as on 2D gel). (B) Two-dimensional gel electrophoresis of [^{32}P]phosphate loaded platelets treated with 100 ng/ml PMA for 5 min, showing a 40–41 kDa (P41) phosphoprotein at same position as the ADP-ribosylated protein. P47 is a major substrate for PMA-stimulated protein kinase C.

within 80% of the prestimulus level after 5 min. At 0.5 U/ml thrombin [^{32}P]G-protein decreased by 65%, but more slowly, reaching its lowest level after about 1 min. Interestingly, the subsequent increase of [^{32}P]G-protein was also much slower, rising to only 45% of the control level in 5 min, suggesting that the rate of reassociation of G-protein subunits ($\alpha\beta\gamma$) was also influenced by the intensity of the stimulus.

Hirudin, which strips thrombin from its receptors [28], rapidly reversed the fall of [^{32}P]G-protein when added 10 s after thrombin. [^{32}P]G-protein returned to control levels in less than 60 s, and in fact rebounded to a level significantly above the prestimulus value by 5 min (fig.2). We conclude that: (a) thrombin decreased ADP-ribosylatable G-protein, probably by causing dissociation of oligomer subunits; (b) despite continuous occupancy of thrombin receptors the level of G-protein progressively returned toward normal at a rate that may be governed by some receptor-initiated reaction; (c) removal of thrombin from its receptors terminated the dissociating stimulus and allowed the restorative process to progress unimpeded at a much higher rate.

Phosphoinositide hydrolysis and Ca^{2+} mobilization caused by thrombin are inhibited by tumor-promoting phorbol diesters and conditions that increase cyclic AMP [18,29–32]. Therefore, we studied the effects of PMA and PGD_2 on thrombin-induced dissociation of G-protein. PMA (100 ng/ml) by itself increased ADP-ribosylatable G-protein by 51–68% within 5 min (table 2), an effect similar to that seen previously in rabbit neutrophil membranes [33]; this increase of ADP-ribosylated protein above control levels (and in thrombin-stimulated cells after addition of hirudin) indicates that about 30% of the total G-protein may be present as free α-subunits in equilibrium with G-protein oligomers, as previously shown in membrane fractions [34]. This

Table 1

Effect of stimulation of platelets with thrombin on basal and pertussis toxin induced [^{32}P]ADP ribosylation of the 40–41 kDa G-protein in membranes isolated from the intact platelets, as described in section 2

Treatment	Amount of ^{32}P-labelled, 40–41 kDa protein, each value relative to its own control (unstimulated)[a]	
	Basal	+ pertussis toxin
Unstimulated platelets	0	1.00 (10)
+ 0.5 U/ml thrombin	0	0.43 ± 0.08 (4)
+ 1.0 U/ml thrombin	0	0.38 (1)
+ 2.0 U/ml thrombin	0	0.38 ± 0.06 (5)

[a] Results expressed as means ± SE, number of experiments in parentheses

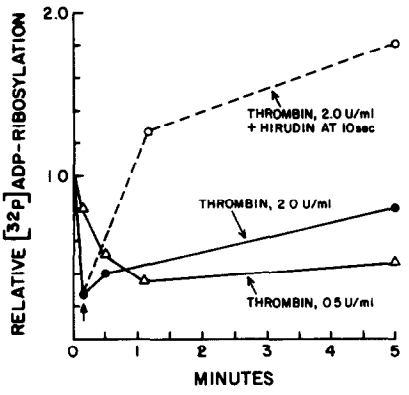

Fig.2. Effect of thrombin on ADP-ribosylatable 40–41 kDa protein of platelet membranes. Platelets (5×10^9/ml) were incubated with thrombin at 37°C for times indicated, and ADP ribosylation measured in isolated membrane fraction as described in section 2.

equilibrium appears to be shifted towards dissociation or reassociation by thrombin and protein kinase C, respectively. Protein kinase C [35] can phosphorylate free α-subunits, and suppress their ability to inhibit adenylate cyclase [34]. In PMA-treated platelets we observed the phosphorylation of a 40–41 kDa polypeptide that migrated identically to the [^{32}P]ADP-ribosylated polypeptide on SDS-PAGE and after isoelectric focusing (fig.1B). The concordance of G-protein phosphorylation with increased ADP-ribosylatable protein suggests that phosphorylation of α-subunits may promote their reassociation with other subunits ($\beta\gamma$) to form oligomers of G-protein. Pretreatment with PMA did not prevent a fall of ADP-ribosylatable G-protein caused by thrombin (table 2). However, because the G-protein was initially elevated 50–60% by PMA, thrombin reduced it only back to the normal prestimulus range. Thus, PMA did not prevent receptor-G-protein interactions but in effect somewhat attenuated the maximal extent of the dissociation of G-protein oligomers normally caused by thrombin. This accords with our previous findings that PMA could only partially inhibit hydrolysis of PIP_2, and indicates that the much stronger inhibition of Ca^{2+} mobilization is caused by some additional action of PMA distal to phospholipase C [29], possibly by activating an IP_3 phosphatase (i.e. P47 protein) [36]. PGD_2, which is a more effective inhibitor of PIP_2 hydrolysis [32], acted quite differently from PMA; in unstimulated platelets G-protein was unaffected, but the fall caused by thrombin was virtually abolished (table 2). This suggests a new mechanism of action for cyclic AMP in platelets, namely to uncouple transduction of the stimulus from the thrombin receptor to G-protein.

Thrombin receptors are involved in the inhibition of adenylate cyclase and the activation of phospholipase C, but no link has been established between G_i, which is coupled to adenylate cyclase, and polyphosphoinositide metabolism. For example, α_2-adrenoreceptors which mediate the inhibition of adenylate cyclase through G_i, do not appear to stimulate phospholipase C. Furthermore, PAF-acether and U44069 stimulate a GTPase activity in platelet membranes that was relative resistant to pertussis toxin [37]. On the other hand, per-

Table 2

Effect of phorbol myristate acetate (PMA) and PGD_2 (+ theophylline) on [^{32}P]ADP ribosylation of 40–41 kDa protein in unstimulated and thrombin-stimulated platelets

	Amount of ^{32}P-labelled, 40–41 kDa protein (each value relative to its own control – unstimulated)	
	Basal	+ pertussis toxin
Untreated platelets	0	1.00
+ PMA (100 ng/ml, 5 min)	0	1.54
+ PMA	0	1.51
+ PMA, then thrombin (2.0 U/ml, 30 s)	0	1.16
+ PMA	0	1.68
+ PMA, then thrombin	0	1.05
Untreated platelets	0	1.00
+ Thrombin (2.0 U/ml, 30 s)	0	0.24
PGD_2/theophylline (2 min), then thrombin (30 s)	0	1.14[a]
+ Thrombin	0	0.37
PGD_2/theophylline, then thrombin	0	0.88

[a] PGD_2 + theophylline by themselves had no effect on ADP ribosylation

tussis toxin suppressed Ca^{2+} mobilization by thrombin in saponin-permeabilized platelets [38]. The pertussis toxin substrate in platelets is present in great excess over G_s [39], and thrombin receptors [41] greatly exceed α_2-adrenoceptors [40] linked to G_i. In neutrophils only 10% of pertussis toxin substrate is detectable by $G_i\alpha$-antiserum, and 90% is immunologically distinct from $G_i\alpha$ or $G_o\alpha$ [42]. These facts suggest that the pertussis toxin substrate in platelets may also be heterogeneous. Further work is necessary to characterize the ADP-ribosylated polypeptides in platelets, and to determine if the effects of thrombin, PMA and PGD_2 on G-protein(s) are relevant to their regulation of phospholipase C activity.

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