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Platelet adenylate cyclase and phospholipase C are affected differentially by ADP-ribosylation

Effects on thrombin-mediated responses

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Thrombin stimulates phospholipase C and inhibits adenylate cyclase in human platelets. We have studied the effect of purified S_1 monomer, the ADP-ribosylating subunit of pertussis toxin, on these receptor-coupled G-protein-dependent activities. ADP-ribosylation of a 41 kDa protein is associated with a marked decrease in the ability of thrombin to inhibit cyclic AMP formation, but has little effect on phospholipase C. Therefore adenylate cyclase and phospholipase C appear to be modulated by different G-proteins.

INTRODUCTION

The incubation of human platelets with thrombin leads to activation of phospholipase C and is associated with the hydrolysis of membrane phosphoinositides and accumulation of inositol trisphosphate and diacylglycerol (Rittenhouse-Simmons, 1979; Rittenhouse & Sasson, 1985; Tarver *et al.*, 1987). Thrombin is also a potent inhibitor of adenylate cyclase in platelets, attenuating the PGE_1-stimulated increase in cyclic AMP (Aktories & Jakobs, 1984). This action of thrombin is mediated by an inhibitory guanine nucleotide-binding protein, G_i (Aktories & Jakobs, 1984). Treatment of platelet membranes and permeabilized platelets with pertussis toxin, which ADP-ribosylates the α-subunit of G_i, uncouples adenylate cyclase from the inhibitory effects of thrombin (Brass *et al.*, 1986; Grandt *et al.*, 1986). Activation of phospholipase C is also considered to be mediated by a G-protein, since permeabilized platelets exposed to stable analogues of GTP exhibit formation of inositol phosphates (Lapetina, 1986) and diacylgycerol (Haslam & Davidson, 1984), and thrombin-induced phospholipase C activation can be inhibited by GDP[S] (Brass *et al.*, 1986). Such phospholipase C-directed G-protein activity has been referred to as G_p (Cockcroft, 1987). Studies with several cell systems have indicated that receptor-stimulated responses are inhibited when such cells are incubated with pertussis toxin (Nakamura & Ui, 1985; Verghese *et al.*, 1985; Paris & Pouyssegur, 1986), implying that G_i may also be G_p. However, pertussis toxin has been shown to inhibit thrombin-induced GTPase activity by only about 30%, perhaps indicating that thrombin stimulates yet another G-protein in human platelets that is relatively resistant to pertussis-toxin treatment (Grandt *et al.*, 1986; Houslay *et al.*, 1986). We have undertaken further studies on the regulation of receptor-coupled enzyme activites by ADP-ribosylation-sensitive G-protein(s). We considered it important to use the purified ADP-ribosylating component (S_1 monomer) of pertussis toxin, rather than the holotoxin, to avoid the effects that the holotoxin's B (binding) subunit appears to exert (Banga *et al.*, 1987; Rosoff *et al.*, 1987). The results show that platelet adenylate cyclase and phospholipase C are affected differentially by exposure of platelets to ADP-ribosylation and that adenylate cyclase is much more sensitive to such treatment.

EXPERIMENTAL

Pertussis toxin was purified, and the S_1 monomer was isolated and inactivated with NEM as presented elsewhere (Banga *et al.*, 1987). Human platelet-rich plasma, free of erythrocytes, was prepared freshly in the presence of aspirin (1 mM) as described by Rittenhouse (1983). Platelets were washed and suspended (1.75×10^9/ml) in buffer, pH 7.1 (Brass *et al.*, 1986). Just before incubation at 37 °C, the platelets were diluted 5-fold with buffer containing 120 mM-KCl, 4 mM-$MgCl_2$, 15 mM-Hepes, 25 mM-NaCl, 1 mM-NaH_2PO_4, 1 mM-EGTA, 280 μM-Ca^{2+}, pH 7.1, and permeabilized with saponin (Brass *et al.*, 1986; Banga *et al.*, 1987) to allow access of S_1 (28 kDa) to substrates.

Detection of ADP-ribosylated protein

Platelets (3.5×10^8/ml) were permeabilized by adding saponin (15 μg/ml) in the presence of [^{32}P]NAD^+ (70 μCi/ml, 1 μM; NEN–Dupont), $NADP^+$ (0.5 mM), ATP (0.5 mM) and S_1 monomer (0–40 μg/ml). $NADP^+$ was included to minimize hydrolysis of NAD^+ by platelets (Rosenthal *et al.*, 1987). The incubations were carried out at 37 °C, and the reaction was stopped after 5–60 min by adding an equal volume of ice-cold 20% (v/v) trichloroacetic acid. The precipitated material was washed with diethyl ether and analysed after one-dimensional SDS/polyacrylamide-gel electrophoresis on 12.5%-acrylamide gels as described by Brass *et al.* (1986). The ADP-ribosylated band was detected by autoradiography and digested with 0.5 ml of H_2O_2 (30%, v/v) at 110 °C before radioactivity counting by

Abbreviations used: G-protein, guanine nucleotide-binding protein; GDP[S], guanosine 5'-[β-thio]diphosphate; NEM, *N*-ethylmaleimide; PGE_1, prostaglandin E_1; PtdOH, phosphatidic acid.

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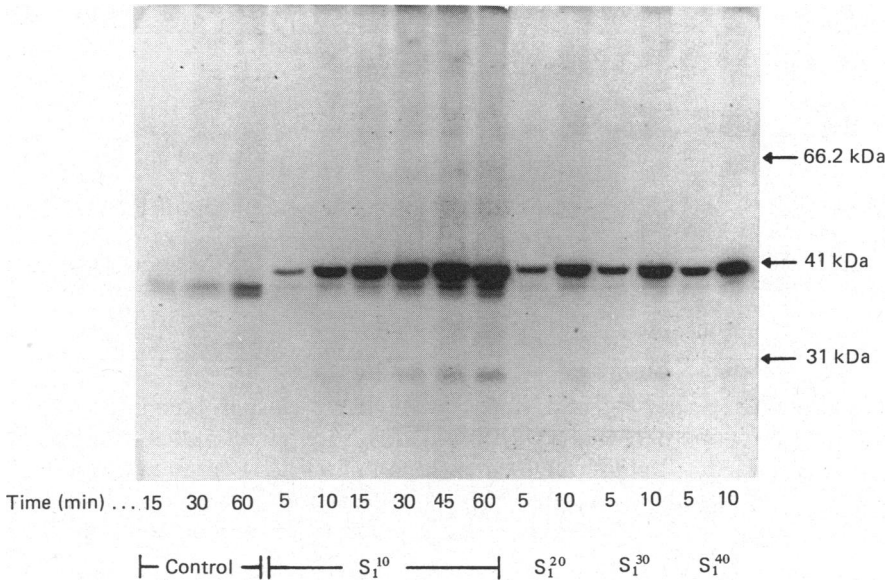

Fig. 1. ADP-ribosylation in permeabilized platelets

Platelets were incubated with saponin (15 μg/ml), [^{32}P]NAD$^+$ (70 μCi/ml; 1 μM), NADP$^+$ (0.5 mM), ATP (0.5 mM) and either buffer (control) or S_1 monomer (Batch 1) with a final concentration of 10 (S_1^{10}), 20 (S_1^{20}), 30 (S_1^{30}) or 40 (S_1^{40}) μg/ml for the time periods indicated. The radiolabelled solubilized proteins were resolved on SDS/12.5%-polyacrylamide gels and observed by autoradiography.

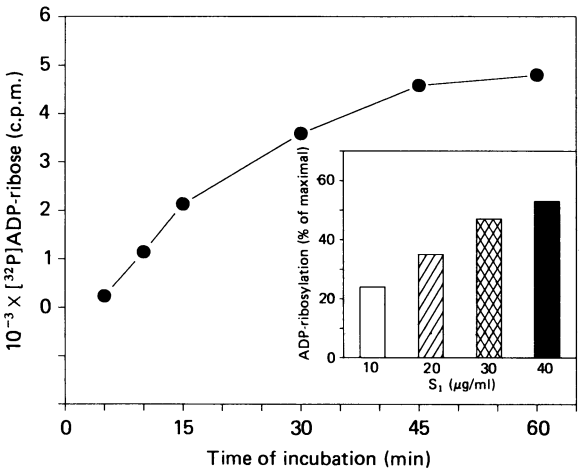

Fig. 2. Time- and dose-dependence of ADP-ribosylation in permeabilized platelets

Platelets were incubated with S_1 (Batch 1; 10 μg/ml) for 5–60 min. Experimental conditions were as described for Fig. 1. The 41 kDa protein band was digested with H_2O_2 before radioactivity counting. Results are c.p.m./4×10^7 platelets. ADP-ribosylation in platelets incubated with increasing concentrations of S_1 for 10 min at 37 °C is shown in the inset as a percentage of maximal ADP-ribosylation; the maximum is considered to be that attained in 60 min with 10 μg of S_1/ml.

scintillation spectrophotometry in 1.0 ml of water + 10 ml of Monofluor (National Diagnostics). Inactivation of S_1 by NEM was confirmed as previously described (Banga *et al.*, 1987).

[^{32}P]NAD$^+$ was separated after the above incubations on 0.1 mm-thick cellulose sheet impregnated with polyethyleneimine (Macherey–Nagel, Düren, Germany), as described by Cassel & Pfeuffer (1978), and its degradation was monitored by counting the radioactivity of the [^{32}P]NAD$^+$ spot after autoradiography.

PtdOH and cyclic AMP formation in permeabilized platelets

Formation of PtdOH (a measure of phospholipase C) and cyclic AMP were measured in permeabilized platelets by conversion of [^{32}P]ATP into respective products, rather than by mass analysis of diacylglycerol, inositol trisphosphate or cyclic AMP. This ensured that only the responses of permeabilized cells (and therefore substrates accessible to S_1) were monitored. S_1 is known to be without an effect on intact platelets (Banga *et al.*, 1987).

Platelets were permeabilized, as described above, by adding saponin (15 μg/ml) at zero time and incubating at 37 °C in the presence of ATP (0.5 mM), NAD$^+$ (0.5 mM) and S_1 monomer (0–30 μg/ml); 9 min later, [γ-^{32}P]ATP (5 μCi/ml; NEN–Dupont) was added and the mixture allowed to equilibrate for 1 min. Platelets were then stimulated with α-thrombin (0.5 unit/ml; courtesy of Dr. Ken Mann, University of Vermont) and the incubation was quenched 2.5 min later with 3.75 vol. of chloroform/methanol/HCl (20:40:1, by vol.). The lipid phase was separated, and [^{32}P]PtdOH was resolved and quantified after t.l.c. (Rittenhouse, 1983).

Adenylate cyclase activity was monitored by conversion of [α-^{32}P]ATP into cyclic [^{32}P]AMP. Saponin-permeabilized platelets (3.5×10^8/ml) were incubated at 37 °C for 9 min, under conditions described above, before addition of [α-^{32}P]ATP (5 μCi/ml; NEN–Dupont). After a further 60 s, buffer or thrombin (0.5 unit/ml) was added, followed by PGE_1 (500 nM) 15 s later. The incubation was continued for 2.5 min and quenched with ice-cold trichloroacetic acid (5%). Cyclic [^{3}H]AMP

(12000 c.p.m.; NEN–Dupont) was added to monitor recoveries, and the cyclic [^{32}P]AMP formed was separated and counted for radioactivity (Salomon *et al.*, 1974).

RESULTS AND DISCUSSION

Incubation of saponin-permeabilized platelets with purified S_1 monomer leads to the ADP-ribosylation of a 41 kDa protein (Fig. 1), which corresponds to the α-subunit of G_i. Significant ADP-ribosylation is observed in a time- and dose-dependent manner (Figs. 1 and 2). More than one speices of ADP-ribosylatable protein may co-migrate in this region; however, we were unable to resolve the 41 kDa band further by gradient-gel electrophoresis. The extent of ^{32}P-monitored ADP-ribosylation is poor when carried out in the absence of $NADP^+$, owing to rapid degradation of [^{32}P]NAD^+ present at low concentrations. The amount of [^{32}P]-NAD^+ falls to about 50% of control within 5 min, and to 15% in 15 min, when incubated with permeabilized platelets in the absence of $NADP^+$, whereas only a 5% decline in [^{32}P]NAD^+ in the presence of $NADP^+$ occurs in 15 min. $NADP^+$ thus slows down the degradation of NAD^+ in the reaction mixture, but does not seem to compete with NAD^+ for pertussis-toxin-catalysed ADP-ribosylation (Rosenthal *et al.*, 1987).

A 30 kDa protein is also found to be ADP-ribosylated in parallel with the 41 kDa band in the presence of S_1 (Fig. 1); however, do do not know whether it is a pertussis-toxin substrate in itself or a proteolytic product of the major 41 kDa substrate(s). Two other proteins, of approx. 39 and 40 kDa, are also labelled when permeabilized platelets are incubated with [^{32}P]NAD^+ and $NADP^+$ in the absence of S_1 (Fig. 1). Such endogenous ADP-ribosylating activity in platelets and erythrocytes has been reported previously (Rosenthal *et al.*, 1987). Although $NADP^+$ efficiently inhibits the endogenous activity associated with platelet membranes, the activity in cytosol is little affected (Rosenthal *et al.*, 1987). Thus the ADP-ribosylation of 39 and 40 kDa proteins that we observe may represent a cytosolic activity in permeabilized platelets. The significance of such endogenous ADP-ribosylation is not yet clear, and requires investigation.

Incubation of permeabilized platelets with S_1 monomer

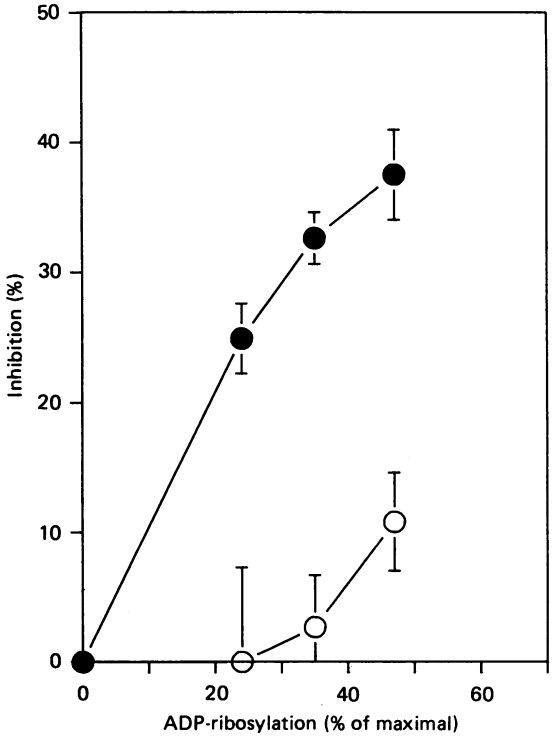

Fig. 3. Effect of ADP-ribosylation on thrombin-mediated responses in permeabilized platelets

Permeabilized platelets, preincubated with S_1 (Batch 1; 0–30 μg/ml) for 10 min at 37 °C, were stimulated with thrombin (0.5 unit/ml) for 2.5 min. The inhibition of thrombin's ability to suppress PGE_1-stimulated cyclic AMP formation (●) and its ability to cause PtdOH accumulation (○) were monitored as described in the Experimental section. Thrombin-mediated inhibition of PGE_1-stimulated cyclic AMP formation in the absence of S_1 was 87.0±0.4% (n = 3). [^{32}P]PtdOH in control platelets was 454±30 c.p.m. (n = 4) and increased to 1734±161 (n = 4) in the thrombin-treated platelets. S_1 had no effect on either PGE_1-stimulated cyclic AMP formation or [^{32}P]PtdOH in control platelets. The results shown are representative of three such experiments. ADP-ribosylation, as % of maximum, was calculated as described for Fig. 2.

Table 1. Effect of ADP-ribosylation on thrombin-mediated inhibition of adenylate cyclase and activation of phospholipase C in permeabilized platelets

Human platelets (3.5×10^8/ml) were permeabilized with saponin in the absence or presence of S_1(Batch 2; 10 or 20 μg/ml) and incubated at 37 °C for 10 min. The PGE_1-mediated increase in cyclic [^{32}P]AMP (control) and the ability of thrombin to inhibit such an increase in platelets incubated with or without S_1 were compared. Cyclic [^{32}P]AMP radioactivity in the absence of PGE_1 was 719±82 c.p.m. In a parallel experiment, [^{32}P]PtdOH formation under basal conditions (control) or with thrombin was also measured in platelets preincubated (10 min) with varied concentrations of S_1 (0–20 μg/ml). Values for radioactivity (c.p.m.) are means±S.D. (n = 3).

	Cyclic [^{32}P]AMP (c.p.m.)	[^{32}P]PtdOH (c.p.m.)	Percentage reversal (cyclic AMP)	Percentage inhibition (PtdOH)
Control	23241±1522	1079±15	–	–
+thrombin (0.5 unit/ml)	7296±562	4350±189	0	0
+S_1 (20 μg/ml)	23088±1421	1084±28	–	–
+S_1 (10 μg/ml)+thrombin	16600±207	4079±32	58	8
+S_1 (20 μg/ml)+thrombin	22018±212	3641±150	92	22

under conditions that lead to ADP-ribosylation of the 41 kDa protein inhibits the ability of thrombin to suppress adenylate cyclase, thereby indicating that G_i is functionally modified by such treatment. The degree of such inhibition is related to the proportion of 41 kDa protein ADP-ribosylated (Fig. 3) and is not observed when S_1 is inactivated with NEM (results not shown). However, thrombin-stimulated phospholipase C activity is not affected by S_1 to a similar extent to adenylate cyclase. Considerable inhibition of thrombin-associated effects on adenylate cyclase is observed before any inhibitory effects on thrombin-activated phospholipase C are expressed (Fig. 3). In another set of experiments performed with a different batch of S_1, phospholipase C activity was inhibited by only 22% when the effects of thrombin in suppressing adenylate cyclase were completely reversed (Table 1). Thus our results indicate that these two activities can be modulated differentially by S_1. The inhibition of phospholipase C activity might become more pronounced if a significantly higher proportion of substrate were to be ADP-ribosylated, as has been achieved in cell-free platelet membrane systems incubated for 30 min with 50 μg of pertussis toxin/ml (O'Rourke *et al.*, 1987). However, increasing the concentration of S_1 beyond 30 μg/ml (the ADP-ribosylating equivalent of about 120 μg of pertussis toxin/ml) was found to increase ADP-ribosylation of permeabilized platelet protein only marginally (Fig. 2, inset). Also, incubation of platelets with saponin for longer than 10 min elevates basal PtdOH concentrations, diminishes the activation of phospholipase C in response to thrombin, and increases the amount of endogenous protein degradation (S. E. Rittenhouse & H. S. Banga, unpublished work). Thus an analysis of the relationship between ADP-ribosylation and inhibition of phospholipase C activation after extended incubations under our conditions does not appear to be feasible.

The differential effects of S_1-catalysed ADP-ribosylation on platelet adenylate cyclase and phospholipase C suggest that G_i is not a 'promiscuous' entity in the platelet, coupled to the thrombin receptor but free to interact with either adenylate cyclase or phospholipase C. Phospholipase C-directed 'G_p' may be quite similar to adenylate cyclase-regulating G_i, possibly differing only with regard to susceptibility to ADP-ribosylation [e.g., via blockage of the target cysteine residue (Magee *et al.*, 1987)]. That phospholipase C and adenylate cyclase are coupled to agonists by two different C-proteins in platelets seems more probable, in light of the following additional observations: (1) adrenaline, which efficiently inhibits adenylate cyclase via G_i (Katada *et al.*, 1984), does not activate platelet phospholipase C (Banga *et al.*, 1986); (2) the thromboxane A_2 analogues U46619 [(15*S*)-hydroxy-11α,9α-(epoxymethano)prosta-5,13-dienoic acid] and ONO 11113 (9,11-epithio-11,12-methano-thromboxane A_2) stimulate a phospholipase C activity in platelets that is dependent on G-protein function (Brass *et al.*, 1987), but are unable to inhibit adenylate cyclase (Brass *et al.*, 1987; H. S. Banga & S. E. Rittenhouse, unpublished work). Clearly, an important future direction for research on stimulus–response coupling in platelets is the isolation and complete characterization of 'G_p'.

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