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Inhibition by cyclic AMP of guanine nucleotide-induced activation of phosphoinositide-specific phospholipase C in human platelets

Yukihiro Yada, Seiji Nagao, Yukio Okano and Yoshinori Nozawa

Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi 40, Gifu 500, Japan

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Phosphoinositide-specific phospholipase C (PLC) activity of human platelet membranes was activated by the nonhydrolyzable guanine nucleotide GTPγS. This activation did not occur in either membranes prepared from dibutyryl cyclic AMP-pretreated platelets (A-membranes) or those prepared from untreated cells and subsequently incubated with cyclic AMP (cAMP) (B-membranes). This cAMP-mediated inhibition was abolished in the presence of inhibitors of cAMP-dependent protein kinase (A-kinase), suggesting that the inhibition was due to phosphorylation of (a) protein component(s). No significant differences were observed in the basal PLC activity and the extent of pertussis toxin-catalyzed ADP-ribosylation among control membranes and the two types of phosphorylated membranes (A- and B-membranes). GTP-binding activities of G_s, G_i and GTP-binding proteins of lower molecular masses were not altered by the phosphorylation of the membranes. These findings suggest that a GTP-binding protein is involved in the GTPγS-mediated activation of PLC and that cAMP (plus A-kinase) inhibits this activation by phosphorylating a membrane protein (probably a 240-kDa protein), rather than the GTP-binding protein or PLC itself. It is likely that this phosphorylation uncouples the GTP-binding protein from PLC.

Phosphoinositide-specific phospholipase C; GTP-binding protein; cyclic AMP-dependent protein kinase; (Human platelet)

1. INTRODUCTION

Like many cells, platelets possess both positive and negative control systems for transduction of extracellular signals. Activation of platelets by excitatory stimuli is accompanied by an increase in intracellular Ca^{2+} that initiates cellular responses [1]. Inhibitory stimuli, on the other hand, provoke an increase in cAMP that inhibits the Ca^{2+}-induced responses. It has been reported that the increase in cAMP prevents the agonist-induced activation of phosphoinositide-specific phospholipase C [2,3]. This inhibition has been thought to be mediated by A-kinase, but its mechanism is only poorly understood.

Accumulating evidence indicates that PLC activity of platelets is regulated by a G-protein [4,5]. The addition of GTP or its non-hydrolyzable analogue GTPγS to permeabilized platelets or membranes prepared therefrom has been shown to enhance the formation of inositol 1,4,5-trisphosphate and diacylglycerol [6]. It has also been reported that treatment of permeabilized platelets with pertussis toxin leads to the abolition of the ability of thrombin to activate cellular responses including phosphoinositide hydrolysis [7]. This ef-

Correspondence address: Y. Nozawa, Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi 40, Gifu 500, Japan

Abbreviations: cAMP, cyclic AMP; dbcAMP, dibutyryl cAMP; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; App(NH)p, adenosine 5′-(β,γ-imido)triphosphate; 8-N_3-GTP, 8-azido-guanosine 5′-triphosphate; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride; G-protein, GTP-binding protein; G_s and G_i, the stimulatory and inhibitory G-proteins, respectively, of adenylate cyclase; A-kinase, cAMP-dependent protein kinase; A-membranes, membranes isolated from dbcAMP-pretreated platelets; B-membranes, membranes prepared from untreated platelets and then incubated with cAMP in the presence of Mg^{2+} and ATP

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fect of the toxin is most likely due to the ADP-ribosylation of a 40–41-kDa G-protein subunit in platelet membranes [8,9]. As for the inhibitory action of cAMP on PLC activation in stimulated platelets, it has been suggested that cAMP (together with endogenous A-kinase) phosphorylates a receptor and reduces the ability of platelets to bind and respond to thrombin [10]. However, since the GTPγS-induced IP_3 formation is inhibited in cAMP-treated platelet membranes, it is likely that (a) certain post-receptor step(s) is (are) affected by cAMP. The present study was undertaken to shed light on the mechanism underlying the cAMP-mediated suppression of GTPγS-induced activation of PLC.

2. MATERIALS AND METHODS

2.1. *Preparation of platelet membranes*

Human platelet membranes were prepared by the method of Katada et al. [11] with slight modifications. A platelet suspension was incubated at 37°C for 5 min in the presence and absence of 1 mM dbcAMP. The platelets were then washed twice with ice-cold Tyrode-Hepes buffer, pH 6.8, containing 10 mM EDTA and resuspended in 20 mM Tris-HCl buffer, pH 6.8, containing 5 mM EDTA. The washed platelets were homogenized in a teflon-glass homogenizer (40 strokes) and the resulting homogenate was centrifuged twice at 20000 × *g* for 25 min at 4°C. The final pellet was suspended in 2–3 vols of Hepes buffer, pH 6.8, containing 1 mM EDTA and 0.1 mM DTT to a protein concentration of 8–11 mg/ml and frozen at −80°C until use. The membrane preparations obtained from untreated and dbcAMP-pretreated platelets were termed control membranes and A-membranes, respectively. B-membranes were obtained by incubating the control membranes with 1 μM cAMP, 20 μM ATP and 5 mM Mg^{2+} at 37°C for 5 min, followed by washing with and resuspending in the same buffer.

2.2. *Assay of PLC activity*

PLC activity was assayed by measuring the formation of [^{3}H]IP_3 from [^{3}H]phosphatidylinositol 4,5-bisphosphate ([^{3}H]PIP_2) as described in [12]. The standard reaction mixture (final volume, 68 μl) contained 25 mM Tris-maleate buffer, pH 5.5, 50 mM KCl, 2 mM EGTA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μM [^{3}H]PIP_2 (15000 cpm) and membranes (1–20 μg protein). For PLC assay of phosphorylated membranes, control membranes were preincubated in the phosphorylation mixture described below except that [γ-^{32}P]ATP was replaced by unlabelled ATP and the mixture was cooled rapidly at 4°C for 2 min. An aliquot of the phosphorylated membrane suspension was added to the standard assay mixture for PLC activity. Protein was determined using a Bio-Rad protein assay kit with bovine serum albumin as a standard.

2.3. *Phosphorylation of platelet membranes*

The reaction mixture (final volume, 200 μl) contained 25 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$ and 20 μM [γ-^{32}P]ATP. Where indicated, 1 μM cAMP, 10 μg of A-kinase purified from bovine heart, 10 μM H-8 or 10 μg protein kinase inhibitor (Type-II) was added. The reaction was run at 30°C for 2–5 min and stopped by heating the mixture at 100°C for 2 min after the addition of 0.5 vol. of SDS-stop solution [150 mM Tris-HCl buffer, pH 6.8, containing 7.5% SDS, 12% glycerol, 4.5% 2-mercaptoethanol (w/v)]. The phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [13]. The gels were then subjected to autoradiography at −80°C for 24–48 h using X-Omat K films.

2.4. *ADP-ribosylation of platelet membranes*

Pertussis toxin-catalyzed ADP-ribosylation of membrane proteins was measured essentially as described previously [14]. The reaction mixture (final volume, 100 μl) contained 20 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM ATP, 3 mM potassium phospho*enol*pyruvate, 10 mM thymidine, 10 μg/ml pyruvate kinase, 1–2.5 μM [α-^{32}P]NAD (1000 Ci/mmol), 5 μg/ml of pertussis toxin preincubated with 10 mM DTT and 1 mM ATP at 30°C for 15 min, and membranes (10–40 μg protein). Incubation was conducted at 30°C for indicated periods of time, and the reaction was terminated by heating the mixture at 100°C for 2 min after the addition of 50 μl of the SDS-stop solution. The mixture was then subjected to SDS-PAGE and ADP-ribosylated proteins were visualized by autoradiography at −80°C for 24–48 h.

2.5. *Photoaffinity labelling with 8-azido-[γ-^{32}P]guanosine 5′-triphosphate*

The standard reaction mixture (final volume, 100 μl) contained 20 mM Mops buffer, pH 7.5, 1 mM EDTA, 100 mM NaCl, 25 mM $MgCl_2$, 1 mM App(NH)p, 1 μM 8-N_3-[γ-^{32}P]GTP and membranes (20 μg) in the presence and absence of 1 mM unlabelled GTP or 1 mM GDP [15]. The mixture was incubated in the dark for 10 min at 4°C and then irradiated for 10 min with a UV lamp (wavelength, 254 nm) from a distance of 1 cm. The mixture was then kept at 100°C for 2 min after the addition of 50 μl of the SDS-stop solution. The labelled proteins were analyzed by SDS-PAGE and visualized by autoradiography.

2.6. *Chemicals and biochemicals*

[^{3}H]PIP_2, [γ-^{32}P]ATP and [α-^{32}P]NAD were purchased from New England Nuclear, 8-N_3-[γ-^{32}P]GTP from ICN, pertussis toxin and H-8 from Seikagaku Kogyo, dbcAMP, GTP, ATP, purified bovine heart A-kinase and protein kinase inhibitor (Type-II) from Sigma, and App(NH)p, Gpp(NH)p, guanosine 5′-(2-*O*-thio)diphosphate (GDPβS) and GTPγS from Boehringer, Mannheim. All other chemicals were of reagent grade.

3. RESULTS AND DISCUSSION

The effects of several nucleotides on PLC activity of human platelet membranes are shown in fig.1. Among the nucleotides tested, the nonhydrolyzable guanine nucleotides GTPγS and Gpp(NH)p were the most potent activators of the enzyme. GTP was also effective, though to a lesser extent. GDPβS repressed the stimulatory effects of GTPγS and Gpp(NH)p. These findings indicate

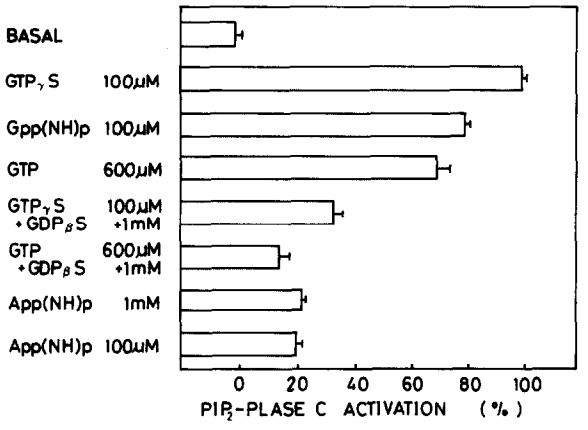

Fig.1. Effects of nucleotides on PLC activity. PLC assays were performed as described in section 2 using 15 μg of platelet membrane protein/assay and 10 min incubation time at 37°C. Results were expressed as percentage of maximal activation obtained by the addition of 100 μM GTPγS. The figure is representative of three different experiments that gave similar results.

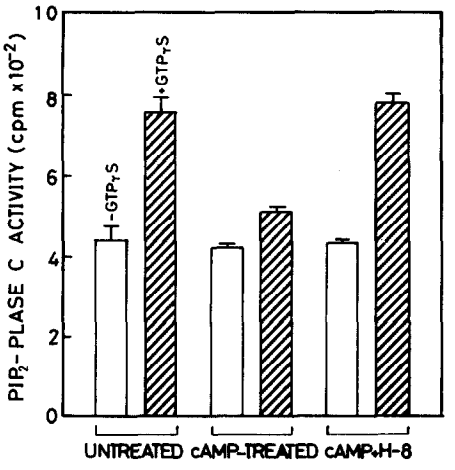

Fig.2. Effects of cAMP and H-8 plus cAMP on GTPγS-stimulated PLC activity. Membranes (200 μg) from untreated cells were preincubated with following reagent(s): UNTREATED, 5 mM $MgCl_2$; cAMP-TREATED, 5 mM $MgCl_2$, 20 μM ATP and 1 μM cAMP; cAMP + H-8, 5 mM $MgCl_2$, 20 μM ATP, 1 μM cAMP and 10 μM H-8. After incubation for 5 min at 30°C, ice-cold Tyrode-Hepes buffer, pH 6.8, containing 1 mM EDTA and 0.1 mM DTT was added to samples. The mixtures were washed twice by centrifugation at 20000 × g for 20 min at 4°C and the final pellet was resuspended in 200 μl of the same Tyrode-Hepes buffer containing EDTA and DTT. 20 μl of the mixtures was used for assay of PLC activity. PLC activity was measured in the presence or absence of 100 μM GTPγS as described in section 2. Results were expressed as means ± SE from three separate experiments.

that a G-protein is involved in this activation, in agreement with previous reports [12,16,17]. Rock et al. [18] have, however, reported that G-protein is not responsible for the activation of PLC in human platelets. The reason for this discrepancy is unclear.

PLC activity of control membranes prepared from untreated platelets was stimulated by GTPγS in a dose-dependent manner, whereas GTPγS did not enhance the enzyme activity in A-membranes prepared from dbcAMP-pretreated cells (not shown). Nevertheless, there were no significant differences between the basal PLC activities of control and A-membranes. B-membranes, prepared from untreated platelets and then incubated with cAMP in the presence of Mg^{2+} and ATP, also showed the same basal PLC activity, which was activated by GTPγS only slightly (fig.2). In the presence of H-8, an inhibitor of A-kinase, however, GTPγS stimulated the PLC activity even in the cAMP-treated membranes (B-membranes) (fig.2). As shown in fig.3, the addition of cAMP alone or cAMP plus purified A-kinase together with ATP and Mg^{2+} to platelet membranes caused the phosphorylation of 22-, 24-, 50- and 240-kDa proteins (lanes A and C), which have been shown to serve as substrates for A-kinase [19]. This phosphorylation was inhibited by H-8 (lanes B and D). Protein kinase inhibitor (Type-II), another A-kinase inhibitor, behaved similarly towards the GTPγS-induced PLC activation and the protein phosphorylation (not shown). It is thus evident that protein phosphorylation by A-kinase is responsible for the inhibition of PLC activation. However, since the basal PLC activities of both A- and B-membranes were practically the same as that of control membranes, it is unlikely that A-kinase phosphorylated PLC directly. In fact, PLC purified from human platelet membranes [20] was not phosphorylated by A-kinase (unpublished). It can be assumed that A-kinase phosphorylates either the G-protein involved or a component functional between the G-protein and PLC and thus uncouples the G-protein from PLC.

ADP-ribosylation of α-subunits of G-proteins by cholera toxin (for G_s) and pertussis toxin (for G_i and G_o) has been widely used to assess the func-

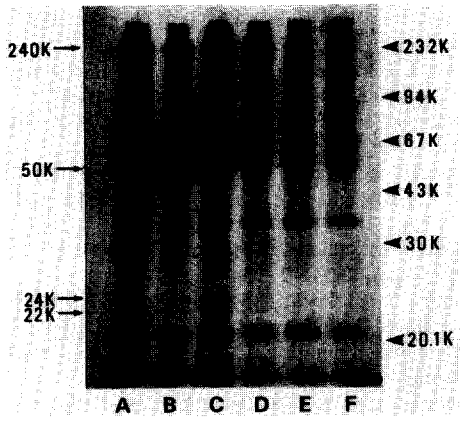

Fig.3. Autoradiograph of SDS-PAGE showing phosphorylation of platelet membrane proteins by A-kinase. Membranes were isolated from untreated platelets. Phosphorylation of the membranes was performed as described in section 2 in the presence of following addition(s): lane A, exogenous A-kinase and cAMP; lane B, exogenous A-kinase, cAMP and H-8; lane C, cAMP; lane D, cAMP and H-8; lane E, none; lane F, H-8. Proteins were separated by SDS-PAGE and phosphorylated proteins were detected by autoradiography. The autoradiographic patterns of 12% SDS-PAGE, representative of three separate experiments, are shown.

tions of G-proteins. Pertussis toxin has been found to block the PLC activation in such cells as neutrophils [21,22] but not in others [23,24]. In the case of platelets, this toxin has been reported to both enhance [25] and inhibit [7] the thrombin-mediated activation of PLC. At any rate, the present study showed that cAMP treatment of human platelet membrane did not affect the pertussis toxin-catalyzed ADP-ribosylation of a 40-kDa (α-) subunit of a G-protein (not shown). Furthermore, it was found that the addition of GTPγS and Mg^{2+} decreased the ADP-ribosylation of the 40-kDa protein to the same extent in control, A- and B-membranes (not shown). Quantitative analysis by the rapid filtration method (using nitrocellulose filters) could confirm that the extents of ADP-ribosylation in control, A- and B-membranes were practically the same with one another (not shown). These results appear to exclude the possibility that cAMP directly affects the G-protein, which is effectively ADP-ribosylated by pertussis toxin.

Recent studies have demonstrated the occurrence in platelet membranes of another family of G-proteins, which have smaller molecular masses (21–29.5-kDa) and are insensitive to the action of pertussis toxin [26,27]. Although the G-protein involved in the PLC activation has not yet been identified in platelets, the possibility exists that the activation of the G-protein is accompanied by an exchange of GDP by GTP, as in the case of the adenylate cyclase system. If this is the case, then it is likely that the cAMP-induced suppression of PLC activation is caused by the cAMP-dependent phosphorylation of the G-protein, leading to the inhibition of this GDP-GTP exchange. It was, therefore, expected that the GTP-binding activity of the G-protein would be attenuated by pretreatment of control membranes with dbcAMP. To test this possibility, photoaffinity labelling of control and A-membranes was conducted with 8-N_3-[γ-^{32}P]GTP as a label. Both types of membranes were incubated with 8-N_3-[γ-^{32}P]GTP in the dark and then photolyzed. App(NH)p was included in the incubation mixture to prevent nonspecific nucleotide binding. As shown in fig.4,

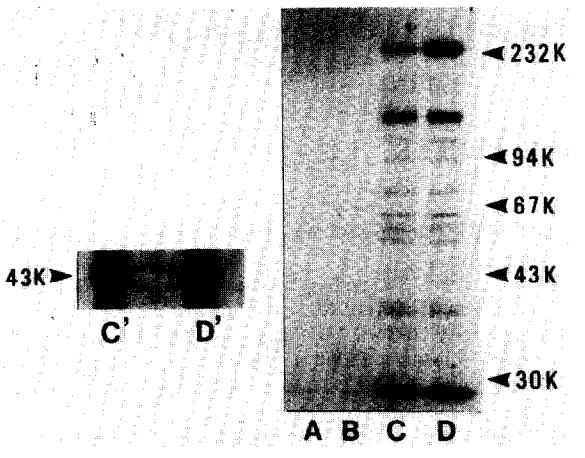

Fig.4. Photoaffinity labelling by 8-N_3-[γ-^{32}P]GTP. Membranes were isolated from untreated control and dbcAMP-pretreated cells, and photoaffinity labelling was performed as described in section 2. Samples are as follows: lane A, membranes incubated with 8-N_3-[γ-^{32}P]GTP in the dark; lane B, membranes incubated with 8-N_3-[γ-^{32}P]GTP and unlabelled 1 mM GTP in the dark and irradiated with UV; lane C, membranes incubated with 8-N_3-[γ-^{32}P]GTP in the dark and irradiated with UV; lane D, dbcAMP-pretreated membranes incubated with 8-N_3-[γ-^{32}P]GTP in the dark and irradiated with UV. Lane C' and lane D' (the same samples of lane C and lane D) were excessively exposed for 5 days. The autoradiographic patterns of 9% SDS-PAGE, representative of three separate experiments, are shown.

we could thus detect the labelling of G_s, G_i (lanes C and C′) and smaller G-proteins of 21–27-kDa (not shown) in control membranes. This labelling was blocked by either GTP (lane B) or GDP (not shown). In A-membranes G_s, G_i and smaller G-proteins were labelled to the same extents as in control membranes (lanes D and D′). It was, however, found that a 240-kDa protein was much more strongly labelled in A-membranes than in control membranes. Since a protein having the same electrophoretic mobility as the 240-kDa protein was phosphorylated by A-kinase (cf. fig.3), it may be concluded that the GTP-binding activity of this protein is enhanced by the A-kinase-catalyzed phosphorylation.

Taken together, the results described above suggest that the cAMP-dependent phosphorylation of the 240-kDa membrane protein is responsible for the suppression of the GTPγS-induced activation of PLC. The phosphorylation seems to increase the capacity of the 240-kDa protein to bind GTP. It is likely that the binding of GTP to this protein somehow uncouples the functional connection between the G-protein involved and PLC.

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