Possible involvement of different GTP-binding proteins in noradrenaline- and thrombin-stimulated release of arachidonic acid in rabbit platelets

Yasuo KAJIYAMA, Toshihiko MURAYAMA,* Yoshihisa KITAMURA, Shin-ichi IMAI and Yasuyuki NOMURA

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

Noradrenaline (NA) stimulated the release of arachidonic acid (AA) from the [³H]AA-labelled rabbit platelets via α_2 -adrenergic receptors, since the effect of NA was inhibited by yohimbine. The stimulatory effect of NA in digitonin-permeabilized platelets was completely dependent on the simultaneous presence of GTP and Ca^{2+} . The NA- and thrombin-stimulated releases of AA were markedly decreased by the prior ADP-ribosylation of the permeabilized platelets with pertussis toxin. Antiserum directed against the pig brain G_o (a GTP-binding protein of unknown function), recognizing both α_{39} and $\beta_{35,36}$ subunits, but not α_{41} , of pig brain, reacted with 41 kDa and 40 kDa bands, with not one of 39 kDa, in rabbit platelet membranes. Anti- G_o antiserum inhibited guanosine 5'-[γ -thio]triphosphate-, AlF_4^- , NA- and thrombin-stimulated AA releases in the membranes. Although the effect of thrombin was inhibited by low concentrations of anti- G_o antiserum, high concentrations of the antiserum was needed for inhibition of the NA effect. Antiserum directed against the pig brain G_i (inhibitory G-protein), recognizing both α_{41} and $\beta_{35,36}$ subunits, but not α_{39} , of pig brain, reacted with the 41 kDa band in platelets. Anti- G_i antiserum inhibited only the effect of NA. Reconstitution of the platelet membranes ADP-ribosylated by pertussis toxin with G_o , not G_i , purified from pig brain restored the thrombin-stimulated release of AA. In contrast, reconstitution of those membranes with G_i , not G_o , restored the NA-stimulated release of AA. These results indicate that different GTP-binding proteins, G_i - and G_o -like proteins, may be involved in the mechanism of signal transduction from α_2 -adrenergic receptors and thrombin receptors to phospholipase A_2 in rabbit platelets.

INTRODUCTION

Platelets undergo aggregation and secretion of granule contents after exposure to adrenaline or noradrenaline (NA). This effect is blocked by α_2 -adrenergic antagonists such as yohimbine, and therefore is assumed to involve the α_2 -adrenergic receptor (Sweatt *et al.*, 1985, 1986a,b; Banga *et al.*, 1986). α_2 -Receptor stimulants and thrombin are shown to cause activation of phospholipase A_2 by a pathway involving Na^+/H^+ exchange (Sweatt *et al.*, 1985, 1986a,b) and to cause inhibition of adenylate cyclase by a pathway involving G_i , the inhibitory GTP-binding protein (Jakobs *et al.*, 1978, 1982). GTP-binding proteins (G-proteins), including G_i , are apparently present in every cell type, and are well known to serve as transducers for specific membrane receptors to intracellular effectors such as adenylate cyclase and phospholipases A_2 and C (Casey & Gilman, 1988; Neer & Clapham, 1988). Much evidence indicates that thrombin receptors are coupled to G-proteins which modulate adenylate cyclase (Aktories & Jakobs, 1984) and phospholipase A_2 (Fuse & Tai, 1987; Nakashima *et al.*, 1987a,b; Crouch & Lapetina, 1988; Kajiya *et al.*, 1989; Murayama *et al.*, 1990a,b) activities in human and rabbit platelets. The G-proteins are heterotrimers consisting of α , β and γ subunits. Recently, it has become clear that there are a number of α subunits, and it has been suggested that this diversity of α subunits is responsible for the specificity of transduction to different effectors (Casey & Gilman, 1988; Neer & Clapham, 1988). It remains to be determined whether involvement of G-proteins is linked to the ability of NA to release arachidonic acid (AA).

With this background, the present studies were designed to address two aspects of the regulation of NA-stimulated phospholipase A_2 activity in rabbit platelets: (a) to determine whether NA-stimulated AA release is, like thrombin-stimulated AA release, mediated by G-proteins, and if so, (b) to determine whether the G-proteins that couple α_2 -adrenergic receptors are the same as those that couple thrombin receptors. The effects of anti- G_o and anti- G_i antisera and purified G_o and G_i proteins on receptor-stimulated AA releases were tested. The results provide evidence that signals from α_2 -adrenergic receptors and thrombin receptors are transduced by different G-proteins to phospholipase A_2 .

MATERIALS AND METHODS

Materials

NA, clonidine, phenylephrine, yohimbine, prazosin, propranolol, human α -thrombin and GTP were purchased from Sigma. Guanosine 5'-[γ -thio]triphosphate (GTP[S]) was from Boehringer. Indomethacin was kindly given by Redary Corp. [⁵,6,8,9,11,12,14,15-³H]AA (80–140 Ci/mmol) was purchased from Amersham Corp. Pertussis toxin, purified from a 2-day culture supernatant of *Bordetella pertussis*, was generously given by Kaken-seiyaku (Kyoto, Japan). Purified pig G_o and G_i were prepared by the methods of Katada *et al.* (1986). Anti- G_o and anti- G_i antisera, which were obtained from mice immunized with purified pig G-proteins, were prepared by the methods of Kitamura *et al.* (1989). All other chemicals used were those described in previous papers (Murayama & Ui, 1985, 1987;

Abbreviations used: AA, arachidonic acid; G-proteins, GTP-binding proteins; G_i , the inhibitory G-protein of adenylate cyclase; G_o , a major pertussis-toxin substrate of brain 'other' than G_i ; GTP[S], guanosine 5'-[γ -thio]triphosphate; NA, noradrenaline.

* To whom correspondence should be addressed.

Kajiyama *et al.*, 1989; Murayama *et al.*, 1990a,b), and were of analytical-reagent grade.

Isolation of rabbit platelets and prior radiolabelling

Blood (60 ml) was obtained from male albino rabbits weighing 2–3 kg. Blood was mixed with 0.2 vol. of ACD buffer (71 mM-citric acid, 85 mM-trisodium citrate, 111 mM-dextrose, pH 5.5) to prevent coagulation. Platelet-rich plasma was obtained by centrifugation at 150 *g* for 10 min, and platelets were prepared by Sepharose 2B chromatography and centrifugation at 1500 *g* for 10 min at room temperature in a modified Tyrode–Hepes buffer (134 mM-NaCl, 12 mM-NaHCO₃, 2.9 mM-KCl, 0.34 mM-Na₂HPO₄, 1 mM-MgCl₂, 10 mM-Hepes, 5 mM-glucose, 0.3 % BSA, pH 7.4). Platelets were incubated for 2 h at 30 °C with [³H]AA (5 µCi/ml) in the presence of 10 µM-indomethacin and then washed with 2 × 20 ml of the Tyrode–Hepes buffer.

Digitonin treatment

The [³H]AA-labelled platelets were incubated for 10 min at 30 °C with 10 µM-digitonin. To avoid platelet aggregation, 5 µM-indomethacin and 10 units of aprotinin/ml were added. This treatment was terminated by dilution with 10 ml of the Tyrode–Hepes buffer. The platelet pellet was prepared and washed twice by centrifugation at 1500 *g* for 10 min at 10 °C.

Reconstitution of the ADP-ribosylated platelet membranes with pig brain G_o or G_i

The [³H]AA-labelled platelets treated with or without pertussis toxin were homogenized by using a Dounce homogenizer. The membranes were prepared and washed twice with the Tyrode–Hepes buffer by centrifugation at 30000 *g* for 15 min at 4 °C. The isolated membranes were suspended at a concentration of 3 mg of protein/ml in the same buffer. Various amounts of pig brain G_o or G_i were added to the ADP-ribosylated rabbit platelet membranes or control membranes. When purified G_i (2 nmol/ml) or G_o (4 nmol/ml) was added, 10–25 µl of the protein [in 20 mM-Tris (pH 8.0)/1 mM-EDTA/1 mM-dithiothreitol/0.5 % CHAPS] was included in this reaction mixture. Final CHAPS concentrations did not exceed 0.05 %, and the possibilities such as effects of the detergent have been excluded. Each mixture was preincubated for 10 min at 30 °C and then used for assay of [³H]AA release without washing.

Assay for [³H]AA release

The intact platelets [(4–5) × 10⁶ cells/tube], the permeabilized platelets [(8–10) × 10⁶ cells/tube] or the reconstituted membranes (50 µg of protein/tube) were resuspended in the Tyrode–Hepes buffer and incubated at 37 °C with the addition of 100 µM-CaCl₂ and further additions shown in Tables and Figures. The reaction time was 7 min for [³H]AA release. The reaction was terminated by addition of 1 ml of the ice-cold Tyrode–Hepes buffer containing 2 mM-EGTA and 5 mM-EDTA followed by centrifugation for 3 min at 8000 *g* for the intact or permeabilized platelets, or at 20000 *g* for the membranes, at 4 °C. The radioactivity in the supernatant was measured.

Preparation of anti-G-protein antisera and immunoblots

G_o and G_i were purified from pig brain by the methods of Katada *et al.* (1986). Fig. 3(b) shows the Coomassie Blue staining patterns on SDS/PAGE of purified G_o and G_i protein preparations. Anti-G_o and anti-G_i antisera were prepared from BALB/c mice injected with pig brain G_o or G_i intraperitoneally as described previously (Kitamura *et al.*, 1989). The specificity and the cross-reactivity of the antisera used are shown in Fig. 3a. The crude membranes of pig brain containing G_o and G_i (Fig. 3a) or rabbit platelet membranes (Fig. 7) were separated by

SDS/PAGE, and then electrophoretically transferred from slab gel to GVHP membranes (Millipore Corp.). After the trans-blotting, the membranes were incubated with phosphate-buffered saline containing 1 % BSA at 4 °C overnight to block non-specific protein binding. The membranes were incubated with the same buffer containing the antiserum (anti-G_o or anti-G_i) at room temperature for 3 h. After being washed with phosphate-buffered saline, the membranes were incubated with second antibody diluted 1:500 (affinity-purified goat anti-mouse IgG, horseradish peroxidase-conjugated; Bio-Rad) in the buffer containing 1 % BSA for 2 h. After washing with the buffer, 0.6 mg of 4-chloro-1-naphthol/ml and 0.02 % H₂O₂ in 10 mM-Tris buffer (pH 7.4) were then added. The protein bands that cross-reacted with antiserum against the subunits of G-proteins could be visually detected after 3–5 min at room temperature, and then the membranes were washed with water to prevent the peroxidase reaction. Anti-G_o antiserum reacted with α subunit (α₃₉) and β subunits of G_o. Anti-G_i antiserum reacted with α subunit (α₄₁) and β subunits of G_i (Fig. 3a).

Data presentation

All experiments were repeated 2–4 times under the same conditions, and were reproducible and in agreement with each other within 20 % error. In some cases, results are means of duplicate or triplicate determinations from a typical experiment.

RESULTS

Effect of NA on AA release

NA caused a dose-related increase in [³H]AA release from the labelled rabbit platelets (Fig. 1a). Addition of the α₂-adrenergic agonist clonidine also caused [³H]AA release, with a maximal release 80–90 % of that obtained with NA. The α₁-adrenergic agonist phenylephrine produced only a small degree of [³H]AA release. The nature of the adrenergic receptor regulating AA release was further studied by using antagonists (Fig. 1b). The ability of NA to cause [³H]AA release was blocked by the addition of the α₂-adrenergic antagonist yohimbine, but not the α₁-antagonist prazosin. The [³H]AA release induced by NA was also not blocked by the β-adrenergic antagonist propranolol or

Fig. 1. Effect of α-adrenergic agents on [³H]AA release

The [³H]AA-labelled platelets were washed with the Tyrode–Hepes buffer and incubated with α-adrenergic agents in the presence of 100 µM-CaCl₂. (a) Platelets were incubated with increasing concentrations of NA (○), clonidine (△) or phenylephrine (□). (b) Platelets were first incubated with yohimbine (▲) or prazosin (■) for 5 min and further incubated with 10 µM-NA. The release of [³H]AA was assayed as described in the Materials and methods section. Each point is the mean of triplicate determinations in one typical experiment. Experiments were replicated twice with similar results.

Fig. 2. GTP-analogue-dependent release of [³H]AA by NA in permeabilized platelets

The labelled platelets were treated with 15 μ M-digitonin as described in the Materials and methods section. The permeabilized platelets were then assayed for [³H]AA release with increasing concentrations of NA (○; a) or GTP[S] (○; b). The assay medium was further supplemented with 10 μ M-GTP (●; a) or 10 μ M-NA (●; b). Each point is the mean of triplicate determinations in one typical experiment. Experiments were replicated twice with similar results.

by indomethacin (results not shown). These results indicate that the effect of NA in rabbit platelets is mediated via α_2 -adrenergic receptors, as in human platelets (Sweatt *et al.*, 1985, 1986a,b; Banga *et al.*, 1986).

Interaction of pertussis-toxin-sensitive G-proteins with α_2 -adrenergic receptors and phospholipase A₂

Various studies, including some from our laboratory, suggest that G-proteins are involved in activation of phospholipase A₂ (Fuse & Tai, 1987; Nakashima *et al.*, 1987a,b; Crouch & Lapetina, 1988; Kajiyama *et al.*, 1989; Murayama *et al.*, 1990a,b). To determine whether or not G-proteins are involved in NA-stimulated AA release, the platelets were incubated with digitonin to permeabilize plasma membranes, and then the effects of NA or GTP analogues in the presence of Ca²⁺ were examined (Fig. 2). NA-stimulated [³H]AA release was not observed in the permeabilized platelets, in contrast with intact platelets. AA release was markedly stimulated by NA when the reaction mixture was supplemented with 10 μ M-GTP. The AA release attained its maximum value of 60–70%, comparable with the maximal value induced by NA in intact platelets. The effect of NA in permeabilized platelets was blocked by yohimbine, but not by prazosin (results not shown). The ED₅₀ value for NA action was not changed by digitonin treatment. Similar experiments were repeated with increasing concentrations of GTP[S] (Fig. 2b). Without receptor stimulation, GTP caused little [³H]AA release. When a hydrolysis-resistant GTP analogue, GTP[S], replaced GTP, AA releases were observed even in the absence of NA, and GTP[S]-induced AA release was enhanced by NA.

Since the results mentioned above suggested the involvement of the G-proteins in NA-stimulated AA release, the effect of pertussis toxin was investigated. It has been reported that pertussis toxin catalysed the ADP-ribosylation of certain G-proteins of platelets, decreasing their ability to couple thrombin receptors to effector proteins such as adenylate cyclase (Aktories & Jakobs, 1984) or phospholipase A₂ (Nakashima *et al.*, 1987a,b; Kajiyama *et al.*, 1989), and to couple α_2 -adrenergic receptors to adenylate cyclase (Jakobs *et al.*, 1978, 1982). In our phospholipase A₂ assay system using permeabilized platelets, pertussis toxin blocked not only thrombin- but also NA-stimulated [³H]AA

Table 1. Pertussis toxin inhibits thrombin- and NA-stimulated [³H]AA release

The labelled platelets were treated with 10 μ M-digitonin plus 100 μ M-NAD⁺, 1 mM-ATP, 10 mM-thymidine and 10 mM-dithiothreitol in the absence (Control) or presence (Pertussis-toxin-treated) of 1 μ g of pre-activated pertussis toxin/ml for ADP-ribosylation of membrane proteins, and then assayed for [³H]AA release as described in the Materials and methods section. The assay mixture was further supplemented with 1 unit of thrombin/ml, 10 μ M-NA and/or 10 μ M-GTP[S]. The data are means $\pm$ S.E.M. from four separate experiments: * Effect of pertussis toxin was significant ($P < 0.01$).

Additions	[³ H]AA released (d.p.m./10 ⁶ cells)	
	Control	Pertussis-toxin-treated
None	75 $\pm$ 12	73 $\pm$ 10
GTP[S]	356 $\pm$ 34	348 $\pm$ 26
Thrombin + GTP[S]	874 $\pm$ 67	398 $\pm$ 36*
NA + GTP[S]	586 $\pm$ 32	321 $\pm$ 28*

release (Table 1). GTP[S]-stimulated [³H]AA release was not modified by pertussis-toxin treatment.

Effects of anti-G_o and anti-G_i antisera

We next tested the capability of anti-G-protein antisera to modulate the phospholipase A₂ activity. Fig. 3(b) shows the Coomassie Blue staining patterns after SDS/PAGE of purified G_o and G_i. Our preparation of G_i in this study is thought to be G_{i1} because the M_r of the α subunit of G_i was 41 000. The specificities of two antisera are shown in Fig. 3(a). Anti-G_o antiserum reacted specifically with α subunit (α_{30}) of G_o, and anti-G_i antiserum reacted with α subunit (α_{41}) of G_i, whereas two antisera both recognized β subunits ($\beta_{35,36}$) in immune blots of

Fig. 3. SDS/PAGE analysis of purified G_o and G_i from pig brain and specific reaction of different antisera with subunits of G_o and G_i by immune-blot analysis

G_i (1.0 μ g, lane 1) and G_o (1.0 μ g, lane 2) were subjected to electrophoresis through 12% polyacrylamide gels, and then stained with Coomassie Blue in (b). In (a), crude membranes from pig brain were subjected to electrophoresis and blotted on to a GVHP sheet (Millipore Corp.) and analysed with the indicated antisera as described in the Materials and methods section. Lane 1, anti-G_i antiserum, and lane 2, anti-G_o antiserum, from mouse, used at a dilution of 1:100. M_r values ($\times 10^{-3}$; 'K') are indicated.

Table 2. Effects of anti- G_o and anti- G_i antisera on GTP[S]- and AlF_4^- -stimulated [3H]AA release

The labelled and permeabilized platelets were preincubated at 30 °C with 40 μ l of control serum or anti- G_o or anti- G_i antisera/ml for 20 min. The platelets were then assayed for [3H]AA release with 10 μ M-GTP[S] or 10 mM-NaF plus 20 μ M- $AlCl_3$. The data are means $\pm$ S.E.M. from three separate experiments: * Effect of anti- G_o antiserum was significant ($P < 0.01$).

Additions	[3H]AA released (d.p.m./ 10^6 cells)		
	Control serum	Anti- G_o antiserum	Anti- G_i antiserum
None	135 $\pm$ 15	132 $\pm$ 12	138 $\pm$ 10
GTP[S]	536 $\pm$ 71	256 $\pm$ 61*	520 $\pm$ 63
AlF_4^-	576 $\pm$ 26	456 $\pm$ 34*	560 $\pm$ 27

G-proteins in crude membranes from pig brain. Any cross-reactivity with γ subunits by the antisera could not be detected. These results show the specificities of the two antisera. The basal [3H]AA release in the presence of Ca^{2+} was not modified by control serum or by the two antisera (Table 2). Both GTP[S]- and AlF_4^- -stimulated AA releases were inhibited by 60 μ l of anti- G_o antiserum/ml. Pretreatment with anti- G_o antiserum inhibited the response to GTP[S] and AlF_4^- by 69% and 23% respectively. In contrast, no inhibition was observed by anti- G_i antiserum.

As illustrated in Fig. 4(a), anti- G_o antiserum was capable of inhibiting thrombin- and NA-stimulated AA release. Maximal inhibition with anti- G_o antiserum was about 80–90% of that obtained with receptor stimulants. Thrombin-stimulated [3H]AA release was more sensitive to anti- G_o antiserum concentration than was NA-stimulated release. Half-maximal inhibition of the thrombin effect and the NA effect occurred with 20 μ l and 40 μ l of the antiserum/ml respectively. Fig. 4(b) shows that anti- G_i antiserum completely inhibited the NA-stimulated AA release. In contrast, the release by thrombin was slightly inhibited by 60 μ g of anti- G_i antiserum/ml. Control serum was ineffective in both cases. Therefore it seems likely that the G-proteins coupled

Fig. 4. Comparison of the inhibitory potency of anti- G_o and anti- G_i antisera on thrombin- and NA-stimulated [3H]AA release

The labelled and permeabilized platelets were preincubated at 30 °C with various concentrations of anti- G_o (a) and anti- G_i (b) antisera for 20 min. The platelets were then assayed for [3H]AA release in the presence of 10 μ M-NA (●) and 1 unit of thrombin/ml (○) with 10 μ M-GTP. [3H]AA release is plotted as a percentage of the control value obtained with control serum, which was 945 $\pm$ 32 (○) or 624 $\pm$ 48 (●) d.p.m./ 10^6 cells. The data are means of two separate experiments or means $\pm$ S.E.M. from three separate experiments. All experiments were performed in duplicate determinations.

Fig. 5. Reconstitution of the ADP-ribosylated membranes with pig brain G_o or G_i and restoration of thrombin effect

The labelled and permeabilized platelets were treated with (●, ▲) or without (○, △) pertussis toxin. Treatment of the platelets with pertussis toxin was carried out as described in the legend to Table 1. The treated platelets were then homogenized and the plasma membranes were isolated. The membranes were suspended at a concentration of 3 mg of protein/ml, and various amounts of pig brain G_o (a) or G_i (b) were added to the membranes. Each mixture was preincubated for 10 min at 30 °C and then incubated for a further 7 min with (○, ●) or without (△, ▲) 1 unit of thrombin/ml in the presence of 10 μ M-GTP. Results are means of two separate experiments. Two experiments were performed in duplicate determinations, and agreed with each other within 10% error.

to α_2 -adrenergic receptors and to thrombin receptors are immunochemically different.

Reconstitution of pig brain G_o or G_i with the ADP-ribosylated rabbit platelet membranes

To examine selective coupling of the purified G_o and G_i with phospholipase A_2 , reconstitution of pig brain G_i or G_o with the ADP-ribosylated platelet membranes was carried out. In the ADP-ribosylated membranes prepared from platelets pretreated with pertussis toxin and digitonin, the thrombin- and NA-stimulated AA releases were markedly decreased compared with that of the control membranes, as shown in Table 1. When G_o was reconstituted with pertussis-toxin-treated membranes, the effect of GTP was slightly increased and the effect of thrombin plus GTP was markedly increased (Fig. 5a). The maximal stimulation of these reactions was elicited by addition of G_o in an amount 80% to the maximal stimulation by thrombin in control (non-treated) membranes without G_o . On the other hand, when G_i was reconstituted with pertussis-toxin-treated membranes, the effects of GTP and thrombin plus GTP were increased only a little (Fig. 5b). However, addition of various amounts of G_i to the pertussis-toxin-treated membranes restored the NA-stimulated AA release in a dose-dependent manner (Fig. 6b). When G_o was reconstituted with pertussis-toxin-treated membranes, the effect of NA plus GTP was increased slightly (Fig. 6a). Addition of G_o or G_i to the control membranes slightly increased the AA release induced by GTP in the absence of receptor stimulants, and the receptor-stimulated reactions induced by thrombin and NA in the presence of GTP.

G-proteins in platelet membranes

The ability of the two antisera against pig brain G-proteins to achieve recognition of proteins within platelet membranes by immunotransfer blotting after SDS/PAGE is illustrated in Fig. 7. In this experiment, crude platelet membranes were incubated with 0.5% cholate (lane 1) or 1.0% cholate (lane 2) to decrease membrane glycoproteins, which abound in platelet membranes

Fig. 6. Reconstitution of the ADP-ribosylated membranes with pig brain G₀ or G_i and restoration of NA effect

Reconstitution of the ADP-ribosylated membranes with pig brain G₀ (a) or G_i (b) was carried out as described in the legend to Fig. 5. Each mixture was incubated for a further 7 min with (○, ●) or without (△, ▲) 10 μM-NA in the presence of 10 μM-GTP. Results are means of two separate experiments. Two experiments were performed in duplicate determinations, and agreed with each other within 10% error.

Fig. 7. Rabbit platelets contain two immunologically distinct G-proteins that react with anti-G₀ antiserum

Platelet membranes [in 20 mM-Tris (pH 7.4)/1 mM-MgCl₂/0.2 mM-EGTA] were incubated with 0.5% cholate (lane 1) or 1.0% cholate (lane 2) at 4°C for 10 min. Membranes were obtained by centrifugation at 35000 g for 15 min at 4°C and suspended in the buffer. These platelet membranes were separated by SDS/PAGE and then electrophoretically transferred to GVHP sheets. The sheets were incubated with anti-G_i antiserum (a) or anti-G₀ antiserum (b) diluted 1:100. The high-M_r band represents non-specific reaction by glycoproteins that are abundant in platelets.

and interfere with immunoblotting. This brief cholate treatment decreased the total pertussis-toxin substrates by about 30–40% (results not shown), but enabled detection of α subunits of G-proteins in immunoblotting, but lost βγ subunits from platelet membranes. Anti-G_i antiserum recognized 41 kDa protein(s) specifically (Fig. 7a). Anti-G₀ antiserum recognized two bands, of 41 kDa and 40 kDa (Fig. 7b). This result shows the existence of two G₀-like proteins which were recognized by anti-G₀ antiserum in rabbit platelet membranes.

DISCUSSION

α₂-adrenergic-receptor-mediated GTP-dependent activation of phospholipase A₂ in permeabilized platelets

The known receptor-coupled events of α₂-adrenergic receptors in platelets are stimulation of the Na⁺/H⁺ antiporter (Sweatt *et al.*, 1985, 1986a,b), inhibition of adenylate cyclase mediated by G_i (Jakobs *et al.*, 1978, 1982) and stimulation of phospholipase A₂ (Banga *et al.*, 1986; Sweatt *et al.*, 1986a,b). Authi *et al.* (1986) reported that the elevation of intracellular free Ca²⁺ concentration by inositol 1,4,5-trisphosphate, a product of the hydrolysis of phosphoinositides by phospholipase C, stimulated AA release. To assess whether phospholipase A₂ stimulation might provide a mechanism for liberation of AA in response to thrombin or NA, we studied the effects of phospholipase inhibitors and the production of lysophospholipids. First, a phospholipase C inhibitor of phosphoinositide hydrolysis (neomycin) and a diacylglycerol lipase inhibitor (RHC 80267) did not decrease receptor-stimulated AA release (Kajiyama *et al.*, 1989). Even if the reaction mixture contained a cyclo-oxygenase inhibitor, indomethacin, NA and thrombin stimulated AA release (results not shown). These data suggest that the release is not catalysed by the sequential actions of those enzymes. Second, 3–6% of the radioactive phosphatidylcholine, not phosphatidylinositol, was decreased and the equivalent amount of lysophosphatidylcholine was increased during a short-term incubation of the labelled platelets with thrombin (Murayama & Ui, 1987; Nakashima *et al.*, 1987b). With NA stimulation, phosphatidylinositol was hydrolysed and lysophosphatidylinositol was accumulated, in addition to the hydrolysis of phosphatidylcholine (results not shown), as previously reported by Sweatt *et al.* (1986a,b). It is considered that NA stimulates phospholipase A₂ directly via α₂-adrenergic receptors, like the stimulatory and inhibitory effects of NA on Na⁺/H⁺ exchange and adenylate cyclase systems. The difference of original phospholipids may contribute to the involvement of distinct G-proteins in thrombin-stimulated and NA-stimulated phospholipase A₂ activation, as discussed below.

In rabbit platelets, NA stimulated AA release via α₂-adrenergic receptors (Fig. 1). In our experiments using permeabilized platelets or plasma membranes, addition of NA induced AA release directly in a GTP-dependent manner (Fig. 2). In permeabilized platelets, NA-stimulated and GTP-dependent AA release was inhibited by the ADP-ribosylation of G-proteins by pertussis toxin (Table 1). These results strongly suggest that pertussis-toxin-sensitive G-proteins are involved in α₂-adrenergic-receptor-stimulated AA releases.

Effect of anti-G₀ and anti-G_i antisera on NA- and thrombin-stimulated AA release

The G-proteins coupled to α₂-adrenergic receptors and to thrombin receptors were both substrates for pertussis-toxin-catalysed ADP-ribosylation. However, G-proteins for two receptor types were apparently different in the following two respects. First, reactivities to two antisera, anti-G₀ and anti-G_i, were not identical for G-proteins coupled to α₂ receptors and thrombin receptors (Fig. 4). Anti-G₀ antiserum effectively decreased both NA- and thrombin-stimulated AA releases. As shown in Fig. 4(a), thrombin-stimulated AA release was markedly inhibited by low concentrations of anti-G₀ antiserum. In the presence of 30 μg of anti-G₀ antiserum/ml, at which NA-stimulated AA release was not inhibited, thrombin-stimulated AA release was inhibited by 60–70%. The NA-stimulated AA release was completely inhibited by preincubation with anti-G_i antiserum, whereas the thrombin-, GTP[S]- and AlF₄⁻-stimulated AA releases were not inhibited by this preincubation (Fig. 4b and Table 2).

The mouse antisera used in the present studies were raised against pig brain pertussis-toxin substrates (Fig. 3). Anti- G_i antiserum reacted with α subunit (α_{41}) of G_i , but not with α subunit (α_{39}) of G_o . Anti- G_o antiserum reacted with α_{39} of G_o , not with α_{41} of G_i . As wide cross-reactivity of antibodies raised against α and $\beta\gamma$ subunits among different species and tissues has been reported (Roof *et al.*, 1985; Gierschik *et al.*, 1986), the antisera could probably cross-react with G-proteins present in rabbit platelets (Fig. 7). GTP[S]- and AlF_4^- -stimulated AA releases were not inhibited by anti- G_i antiserum, and the inhibition of guanine-nucleotide- or fluoride-stimulated AA releases by anti- G_o antiserum was relatively smaller than the receptor-mediated release in our experiments (Table 2). It is not clear why the function associated with G-proteins should have different sensitivity to anti-G-protein antisera. One possible explanation for these observations is that our antisera, especially anti- G_i antiserum, may be directed against the domains of G-proteins to which receptors are coupled. This remains to be determined.

Reconstitution of membrane phospholipase A_2 with purified G_o and G_i

Second respect is that the degrees of restoration by reconstitution of membranes ADP-ribosylated by pertussis toxin with G_o or G_i , purified from pig brain, was not identical for G-proteins coupled to two types of receptor (Figs. 5 and 6). We demonstrated functional coupling between the exogenous G_o protein and the thrombin receptor and between the exogenous G_i protein and the α_2 -adrenergic receptor, after the ADP-ribosylation of their endogenous α subunits by pertussis toxin. Thrombin or NA stimulated AA release in preparations reconstituted with G_o or G_i respectively. In contrast, the GTP-stimulated AA release in control and ADP-ribosylated membranes, and the NA- and thrombin-stimulated GTP-dependent AA release in control membranes, were increased slightly by addition of exogenous G_o or G_i .

There are reports that the membrane (Kim & Neubig, 1987; Kitamura & Nomura, 1987) and purified (Cerione *et al.*, 1986) α_2 -adrenergic receptor, purified muscarinic receptor (Haga *et al.*, 1985; Kurose *et al.*, 1986), the membrane chemotactic-peptide receptor (Kikuchi *et al.*, 1986) and purified μ opioid receptor (Ueda *et al.*, 1988) were reconstituted with purified G_o and G_i . In many cases, the efficiency of these two G-proteins is equal (Cerione *et al.*, 1986; Kikuchi *et al.*, 1986; Kim & Neubig, 1987; Ueda *et al.*, 1988). Our results demonstrate for the first time functional evidence for the involvement of different G-proteins in coupling α_2 -adrenergic receptor and thrombin receptor in a transmembrane signalling pathway that mediates the stimulation of phospholipase A_2 in platelets.

Role of G-proteins in physiological conditions of platelets

These results presented here clearly indicate that at least two different G-proteins are involved respectively to couple functionally the thrombin receptor and α_2 -adrenergic receptor to the phospholipase A_2 . In fact, the antiserum against G_i (probably G_{i1}) reacted with the α_{41} subunit of G_i , and the antiserum against G_o reacted with two G-proteins (α_{41} and α_{40}), at least in rabbit platelet membranes (Fig. 7). However, it cannot be concluded which transducer, G_o or G_i , may be involved *in vivo* in this coupling in rabbit platelets. The amount of G_o or G_i used in our experiments was 0.1–0.2 μM , a concentration much higher than usual for reconstitutions (Cerione *et al.*, 1986; Kurose *et al.*, 1986; Kitamura & Nomura, 1987; Ueda *et al.*, 1988). The reason is not clear. The ADP-ribosyltransferase activity may remain in pertussis-toxin-treated membranes. Nozawa and collaborators have reported that two G-proteins serving as the substrate of

pertussis toxin, G_{i1} consisting of α_{41} and $\beta\gamma$ subunits, and G_{i2} consisting of α_{40} and $\beta\gamma$ subunits, and not G_o , were purified from human platelet membranes (Nagata *et al.*, 1988). They show that the antibody raised against rat brain α subunit of G_o reacted with pig brain α subunit of G_o , but not with human platelet α subunit of G_{i2} (40 kDa), and suggest that α subunit of G_{i2} was immunochemically different from that of brain G_o . It would be interesting to see whether G_{i2} can restore thrombin-stimulated AA release in pertussis-toxin-treated membranes, as a substitute for G_o .

The precise mechanism of G-proteins to control the phospholipase A_2 activity remains to be established. Although it is generally agreed that G-proteins have three subunits (α , β , γ), the role of subunits in which phospholipase A_2 is regulated is not determined. In the photoreceptor system, the $\beta\gamma$ subunits have been proposed to be stimulatory in phospholipase A_2 (Fung, 1983; Jelsema & Axelrod, 1987), and the $\beta\gamma$ subunits activated the muscarinic K^+ channel in heart (Logothetis *et al.*, 1987). As shown in Fig. 3, both anti- G_o and anti- G_i antisera reacted not only with α subunits but also with β subunits. It is suggested that the $\beta\gamma$ subunits of G-proteins, including G_o and G_i , are the same polypeptide or share a common antigenic determinant(s) (Casey & Gilman, 1988; Neer & Clapham, 1988). If stimulation of phospholipase A_2 activity in platelets was mediated by the $\beta\gamma$ subunits of G-proteins, as in rod outer segments, the antisera used in our experiments could inhibit both α_2 -adrenergic and thrombin-receptor-stimulated AA releases. However, the result is that the AA releases by two types of receptor stimulant were blocked by different antisera. In addition, anti- $\beta\gamma$ antiserum directed against the pig brain $\beta\gamma$ subunits of G-proteins did not inhibit basal and receptor-mediated GTP-dependent AA releases from rabbit platelets (results not shown). An important step for future study will be to identify the roles of subunits.

This work was supported by research grants from the Scientific Research Fund of the Ministry of Education, Science, and Culture, Japan, and from the Uehara Memorial Foundation, Japan.

REFERENCES

- Aktories, K. & Jakobs, K. H. (1984) *Eur. J. Biochem.* **145**, 333–338
- Authi, K. S., Evenden, B. J. & Crawford, N. (1986) *Biochem. J.* **233**, 709–718
- Banga, H. S., Simons, E. R., Brass, L. F. & Rittenhouse, S. E. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9197–9201
- Casey, P. J. & Gilman, A. G. (1988) *J. Biol. Chem.* **263**, 2577–2580
- Cerione, R. A., Regan, J. W., Nakata, H., Codina, J., Benovic, J. L., Gierschik, P., Somers, R. L., Spiegel, A. M., Birnbaumer, L., Lefkowitz, R. J. & Caron, M. G. (1986) *J. Biol. Chem.* **261**, 3901–3909
- Crouch, M. F. & Lapetina, E. J. (1988) *Biochem. Biophys. Res. Commun.* **153**, 21–30
- Fung, B. K.-K. (1983) *J. Biol. Chem.* **258**, 10495–10502
- Fuse, I. & Tai, H. H. (1987) *Biochem. Biophys. Res. Commun.* **146**, 659–665
- Gierschik, P., Falloon, J., Milligan, G., Pines, M., Gallin, J. & Spiegel, A. (1986) *J. Biol. Chem.* **261**, 8058–8062
- Haga, K., Haga, T., Ichijima, A., Katada, T., Kurose, H. & Ui, M. (1985) *Nature (London)* **316**, 731–733
- Jakobs, K. H., Saur, W. & Schultz, G. (1978) *FEBS Lett.* **85**, 167–170
- Jakobs, K. H., Lasch, P., Minuth, M., Aktories, K. & Schultz, G. (1982) *J. Biol. Chem.* **257**, 2829–2833
- Jelsema, C. L. & Axelrod, J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3623–3627
- Kajiya, Y., Murayama, T. & Nomura, Y. (1989) *Arch. Biochem. Biophys.* **274**, 200–208
- Katada, T., Oinuma, M. & Ui, M. (1986) *J. Biol. Chem.* **261**, 8182–8191
- Kikuchi, A., Kozawa, O., Kaibuchi, K., Katada, T., Ui, M. & Taki, Y. (1986) *J. Biol. Chem.* **261**, 11558–11562

- Kim, M. H. & Neubig, R. R. (1987) *Biochemistry* **26**, 3664–3672
- Kitamura, Y. & Nomura, Y. (1987) *J. Neurochem.* **49**, 1894–1901
- Kitamura, Y., Mochii, M., Kodama, R., Agata, K., Watanabe, K., Eguchi, G. & Nomura, Y. (1989) *J. Neurochem.* **53**, 249–257
- Kurose, H., Katada, T., Haga, T., Haga, K., Ichiyama, A. & Ui, M. (1986) *J. Biol. Chem.* **261**, 6423–6428
- Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J. & Clapham, D. E. (1987) *Nature (London)* **325**, 321–326
- Murayama, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 7226–7233
- Murayama, T. & Ui, M. (1987) *J. Biol. Chem.* **262**, 5522–5529
- Murayama, T., Kajiyama, Y. & Nomura, Y. (1990a) *J. Biol. Chem.* **265**, 4290–4295
- Murayama, T., Kajiyama, Y., Takahashi, A. & Nomura, Y. (1990b) *Arch. Biochem. Biophys.* **276**, 146–152
- Nagata, K., Katada, T., Tohkin, M., Itoh, H., Kaziro, Y., Ui, M. & Nozawa, Y. (1988) *FEBS Lett.* **237**, 113–117
- Nakshima, S., Hattori, H., Shirato, L., Takenaka, A. & Nozawa, Y. (1987a) *Biochem. Biophys. Res. Commun.* **148**, 971–978
- Nakashima, S., Tohmatsu, T., Hattori, H., Suganuma, A. & Nozawa, Y. (1987b) *J. Biochem. (Tokyo)* **101**, 1055–1058
- Neer, E. J. & Clapham, D. E. (1988) *Nature (London)* **333**, 129–134
- Roof, D. L., Applebury, M. A. & Sternweis, P. C. (1985) *J. Biol. Chem.* **260**, 16242–16249
- Sweatt, J. D., Johnson, S. L., Cragoe, E. J. & Limbird, L. E. (1985) *J. Biol. Chem.* **260**, 12910–12919
- Sweatt, J. D., Blair, I. A., Cragoe, E. J. & Limbird, L. E. (1986a) *J. Biol. Chem.* **261**, 8660–8666
- Sweatt, J. D., Connolly, T. M., Cragoe, E. J. & Limbird, L. E. (1986b) *J. Biol. Chem.* **261**, 8667–8673
- Ueda, H., Harada, H., Kozaki, M., Katada, T., Ui, M., Satoh, M. & Takagi, H. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7013–7017

Received 2 March 1990/20 April 1990; accepted 4 May 1990