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Functional interaction of prostaglandin E receptor EP_3 subtype with guanine nucleotide-binding proteins, showing low-affinity ligand binding

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The functional interaction of prostaglandin E (PGE) receptor EP_3 subtype with GTP-binding proteins (G proteins) was characterized in the membranes prepared from mouse EP_3 receptor cDNA-transfected Chinese hamster ovary cells. PGE_2 inhibited forskolin-stimulated adenylate cyclase activity in CHO cells expressing EP_3 receptor and this inhibition was abolished by pertussis toxin (PT) treatment. The PGE_2 binding to the membranes was increased by GTPγS, and PT treatment also increased the binding activity to the same level as that increased by GTPγS, but the sensitivity of GTPγS was lost. Reconstitution with PT-sensitive G proteins into the ADP-ribosylated membranes reduced the PGE_2 binding activity with the following preference: Gi1 = Gi2 > Gi3 > Go, but GTPγS completely blocked the reduction by G proteins. The G-protein-induced reduction of the binding was due to the increase in K_d without the change of B_{max}, and due to suppression of association rate. [^{3}H]PGE_2-bound EP_3 receptor solubilized from the ADP-ribosylated membranes in the presence or absence of GTPγS was eluted at the position of M_r = approx. 60 kDa, similar to the relative molecular mass of EP_3 receptor deduced from its amino acid sequence. In contrast, [^{3}H]PGE_2-bound receptor solubilized from Gi2-reconstituted membranes was eluted at the position of M_r = approx. 130 kDa, corresponding to the M_r of the complex of EP_3 receptor and Gi2, but GTPγS shifted the position of its elution from M_r = 130 to 60 kDa. Furthermore, addition of PGE_2 stimulated the GDP release from G proteins reconstituted into the ADP-ribosylated membranes, and PGE_2 inhibited forskolin-stimulated adenylate cyclase activity in G-protein-reconstituted membranes with a selectivity order of Gi1 = Gi2 > Gi3 > Go. These results indicate that EP_3 receptor can functionally couple to PT-sensitive G proteins and unusually the complex form with G proteins has low affinity for the ligand but the form not associated with G proteins has high affinity.

Introduction

Prostaglandin E_2 (PGE_2) produces a broad range of biological actions in diverse tissues through its binding to specific receptors on plasma membranes [1,2]. PGE receptors are pharmacologically subdivided into three subtypes, EP_1, EP_2, and EP_3 [3,4]. Among these subtypes, the EP_3 receptor has been suggested to be involved in inhibition of gastric acid secretion [5], modulation of neurotransmitter release in central and peripheral neurons [6], inhibition of lipolysis in adipose tissue [7], and inhibition of sodium and water reabsorption in kidney tubulus [8,9]. Recently, it has been proposed that PGE_2 acts through receptors that are directly coupled to specific G proteins and that the biochemical outcome of receptor occupancy depends on the nature of the G protein with which the occupied receptor is able to interact [10,11]. In those tissues possessing EP_3 receptor, PGE_2 has been shown to attenuate hormone-stimulated synthesis of cAMP, suggesting that EP_3 receptor exerts above-mentioned functions through Gi [4]. Actually in canine renal outer medulla, PGE_2 receptor was co-solubilized with a pertussis toxin (PT)-sensitive G protein, possibly Gi, using digitonin [12]. Concerning interaction of receptors with

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Abbreviations: PGE_2, prostaglandin E_2; G protein, heterotrimeric GTP-binding protein; GTPγS, guanosine 5′-*O*-(thiotriphosphate); CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PT, pertussis toxin.

G proteins, it has been demonstrated that agonists are capable of inducing or stabilizing a high-affinity form of the receptor, consisting of a complex of agonist, receptor and G protein, and that GTP causes the dissociation of the G protein from the receptor, leading to the transition of the receptor to a form that displays low-affinity agonist binding in various hormone receptors [13–15]. In contrast, GTP stimulated the PGE_2 binding activity to the PGE_2 receptor in canine renal outer medulla [12] and hamster adipocyte [16], indicating that the interaction of EP_3 receptor with the G protein is somewhat different from that of other hormone receptors.

Recently, we have cloned a cDNA for the mouse EP_3 receptor, which belongs to a G-protein-coupled rhodopsin-type receptor, and we stably expressed the EP_3 receptor by DNA transfection into Chinese hamster ovary (CHO) cells [17]. We report here that EP_3 receptor can couple to various PT-sensitive G proteins with a selectivity order of Gi1 = Gi2 > Gi3 > Go, and unusually the receptor has low affinity for the ligand in the complex form with G proteins but high affinity in the form not associated with G proteins, and GTP mediates the transition from the low affinity form to the high affinity one.

Materials and Methods

Materials

[5,6,8,11,12,14,15-^{3}H]PGE_2 (200 Ci/mmol), [8,5′-^{3}H]GDP (35.4 Ci/mmol), and [α-^{32}P]NAD (30 Ci/mmol) were purchased from Du Pont-New England Nuclear. ^{125}I-Labeled cyclic AMP assay system was obtained from Amersham; PGE_2 from Funakoshi Pharmaceuticals (Tokyo, Japan); forskolin from Sigma; pertussis toxin from Seikagaku Kogyo (Tokyo, Japan); GTPγS and GTP from Boehringer-Mannheim; Superose 12 HR 10/30 column from Pharmacia LKB Biotechnology. Gi1 and Go were purified from bovine brain according to the method of Katada et al. [18]. Gi2 and Gi3 were purified from bovine spleen according to the method of Morishita et al. [19]. The purified G proteins appeared to be homogeneous and single entities from the SDS-10% PAGE of proteins visualized by staining with Coomassie brilliant blue.

Reconstitution of G proteins into the ADP-ribosylated membranes

CHO (dhfr$^-$) cells stably expressing EP_3 receptor [17] were exposed to 10 ng/ml PT for 9 h. The harvested cells were homogenized using a Dounce homogenizer in 10 vols. of 20 mM Tris-HCl (pH 7.5), containing 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 0.25 M sucrose. After centrifugation at $800 \times g$ for 10 min, the supernatant was further centrifuged at $300\,000 \times g$ for 20 min. The pellet was washed and suspended in 20 mM Tris-HCl (pH 7.5), containing 10 mM $MgCl_2$, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride to the protein concentration of 10 mg/ml, and then used as the ADP-ribosylated membranes.

G proteins in 1 μl of 20 mM Tris-HCl (pH 8.0), containing 1 mM EDTA, 1 mM dithiothreitol and 1 mM CHAPS were incubated for 1 h at 0°C with 100 μl of the ADP-ribosylated membranes (20 μg). After collection of the membranes by centrifugation for 20 min at $300\,000 \times g$, the reconstituted membranes were resuspended in 100 μl of the respective assay buffer.

Measurements of cAMP formation

Cyclic AMP levels in cells were determined as reported previously [20]. The receptor-expressing cells cultured in 24-well plates ($5 \cdot 10^5$ cells/well) were washed twice with 1 ml of Hepes-buffered saline solution containing 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, and 15 mM Hepes (pH 7.4), and preincubated for 10 min. Reactions were started by the addition of test agents along with 1 mM 3-isobutyl-1-methylxanthine. After incubation for 10 min at 37°C, reactions were terminated by the addition of 10% trichloroacetic acid. Content of cAMP in the cells was measured by the cAMP radioimmunoassay kit.

Adenylate cyclase activity was measured essentially as described previously [21]. The standard assay mixture contained 1 mM EDTA, 5 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 0.5 mM ATP, and 20 μg of the reconstituted membranes in 100 μl of 50 mM Hepes (pH 7.4). Reactions were started by the addition of the membranes, carried out for 10 min at 30°C, and terminated by the addition of 10% trichloroacetic acid. The cAMP formed was measured by the cAMP radioimmunoassay kit.

PGE_2 binding assay

The standard assay mixture contained 2 nM [^{3}H]PGE_2 and 20 μg of the reconstituted membranes in 100 μl of 20 mM Mes (pH 6.0), containing 1 mM EDTA, 10 mM $MgCl_2$ (buffer A). After incubation for 1 h at 30°C, the reaction was terminated by the addition of 2.5 ml of ice-cold buffer A, after which the mixture was rapidly filtered through a Whatman GF/C glass filter (Φ 2.4 cm). The filter was then washed four times with 2.5 ml of ice-cold buffer A, and the radioactivity associated with the filter was measured by scintillation counting using a toluene solution containing 33% (v/v) Triton X-100 and 0.5% 2,4-diphenyloxazole. Nonspecific binding was determined using a 1000-fold excess of unlabeled PGE_2 in the incubation mixture. The specific binding was calculated by subtracting the nonspecific binding from the total binding.

Measurement of GDP release from G proteins reconstituted into the ADP-ribosylated membranes

G proteins were labeled with 10 μM [^{3}H]GDP in the presence of 10 mM $MgCl_2$ at 25°C for 8 h as described previously [22]. After the labeled G proteins had been chromatographed through PD-10 column (Pharmacia LKB Biotechnology) with 20 mM Tris-HCl (pH 8.0), containing 1 mM EDTA, 1 mM dithiothreitol, 0.1 M NaCl, and 1 mM CHAPS, they were reconstituted into the ADP-ribosylated membranes to G protein concentration of 10 pmol/mg of the membrane protein. After the reconstituted membranes (20 μg) had been incubated for 10 min at 30°C with PGE_2 in 100 μl of 20 mM Tris-HCl (pH 7.4), containing 1 mM EDTA, 1 mM $MgCl_2$, and 1 μM GTP, the reaction was terminated by the addition of 2.5 ml of ice-cold 100 mM Tris-HCl (pH 8.0), containing 25 mM $MgCl_2$, 0.1 M NaCl and 1 mM EDTA, after which the mixture was rapidly filtered through a Whatman GF/C glass filter (Φ 2.4 cm). The filter was then washed four times with 2.5 ml of the same buffer, and the radioactivity associated with the filter was measured by scintillation counting.

Gel filtration column chromatography

Samples were chromatographed on a Superose 12 HR 10/30 column at the flow rate of 0.5 ml/min at 4°C, using a fast protein liquid chromatography system. The mobile phase consisted of 0.1 M NaCl and 10 mM CHAPS in buffer A. Fractions of 0.2 ml were collected. The column was calibrated with protein standards of known molecular weights: catalase (M_r = 232 kDa), lactate dehydrogenase (M_r = 144 kDa), bovine serum albumin (M_r = 67 kDa) and ovalbumin (M_r = 43 kDa).

Miscellaneous

[^{32}P]ADP-ribosylation of G proteins in the membranes was performed essentially as described previously [23,24]. Protein concentrations were determined by the method of Lowry et al. [25] using bovine serum albumin as a standard.

Results

Effects of ADP-ribosylation with PT on PGE_2-induced inhibition of cAMP accumulation and PGE_2 binding in CHO cells stably expressing EP_3 receptor

We previously reported PGE_2-induced inhibition of cAMP accumulation in CHO cells stably expressing EP_3 receptor [17]. For further characterization of this effect of PGE_2, we examined the effects of ADP-ribosylation with PT on PGE_2-induced inhibition of cAMP accumulation and PGE_2 binding. When CHO cells were cultured in the presence of increasing concentrations of PT, there was progressive inhibition of the subsequent ADP-ribosylation of a 40-kDa G protein with the toxin in the cells (Fig. 1A). This result indicates that the PT-sensitive G protein was effectively

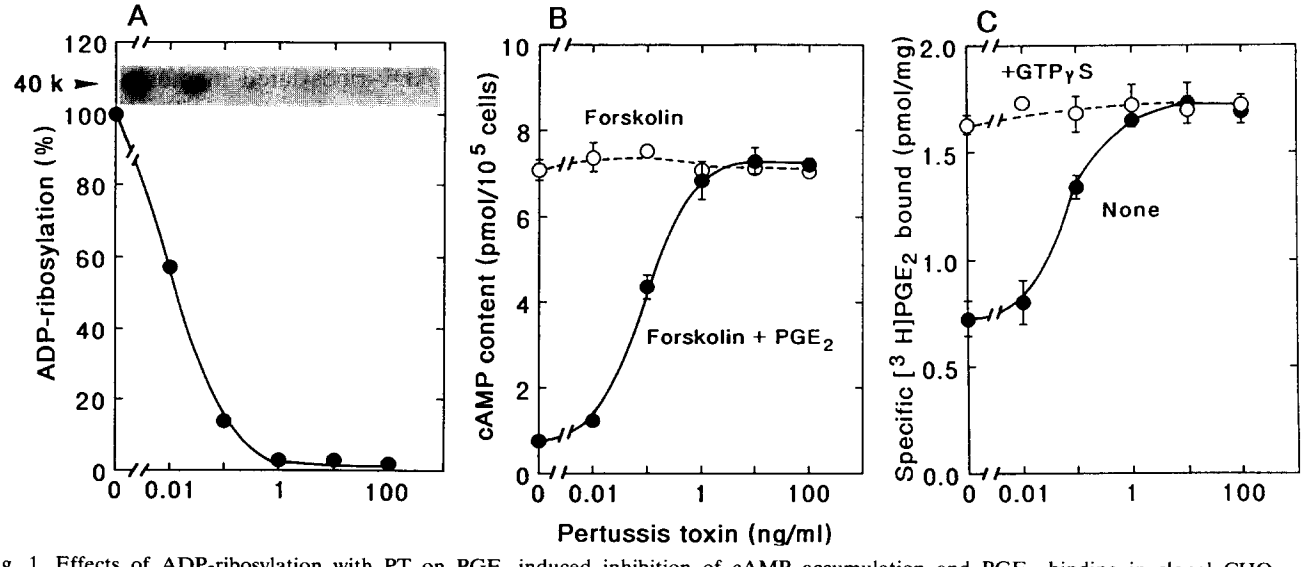

Fig. 1. Effects of ADP-ribosylation with PT on PGE_2-induced inhibition of cAMP accumulation and PGE_2 binding in clonal CHO cells expressing EP_3 receptor. CHO cells stably expressing EP_3 receptor were cultured in the presence of the indicated concentrations of PT for 9 h. (A) After the membranes of these cells had been [^{32}P]ADP-ribosylated with 50 μg/ml preactivated PT, they were subjected to SDS-10% PAGE, followed by autoradiography. The radioactivities of Gi proteins corresponding to an apparent molecular weight of 40000 were determined as described under Materials and Methods. The values are expressed as a percentage of the control (4408 cpm) obtained with the membranes of cells not treated with PT. (B) Cells were incubated for 10 min at 37°C with 1 μM forskolin in the presence (•) or absence (○) of 0.1 μM PGE_2, and cAMP contents accumulated were determined as described under Materials and Methods. (C) The membranes of these cells were assayed for [^{3}H]PGE_2 binding activity in the presence (○) or absence (•) of 100 μM GTPγS as described under Materials and Methods. The values shown are the means ± S.E. for triplicate experiments.

ADP-ribosylated by the prior exposure of the cells to these increasing concentrations of PT. As shown in Fig. 1B, the inhibition of forskolin-induced cAMP accumulation by PGE_2 was attenuated by PT treatment in a dose-dependent manner, and completely suppressed by 10 ng/ml PT. GTPγS, a nonhydrolyzable GTP analogue, potentiated the specific PGE_2 binding activity in the control CHO cell membranes (Fig. 1C). The PT treatment dose-dependently potentiated it and its level maximally reached the level potentiated by GTPγS in the control cell membranes, but GTPγS did not affect the binding. These results suggest that EP_3 receptor is linked to adenylate cyclase via a PT-sensitive G protein and the G-protein-uncoupled form of EP_3 receptor has the higher binding activity than the G-protein-coupled form.

Reconstitution of EP_3 receptor with G proteins

To elucidate which type of PT-sensitive G protein can be coupled to EP_3 receptor, we carried out reconstitution of EP_3 receptor with various PT-sensitive G proteins in the ADP-ribosylated membranes. As shown in Fig. 2A, the specific PGE_2 binding to the membranes was markedly suppressed by the reconstitution of G proteins in the order of Gi1 = Gi2 > Gi3 > Go (IC_{50} values were determined to be 0.5, 0.5, 3, and 20 pmol/mg of the membrane protein, respectively). However, GTPγS completely blocked the suppression by these G proteins (Fig. 2B). Furthermore, GppNHp, GTP or GDP also blocked the suppression in that order, but ATP did not at all (data not shown). On the other hand, when these G proteins were first ADP-ribosylated with PT and then reconstituted into the ADP-ribosylated membranes, they were unable to suppress the PGE_2 binding activity (data not shown).

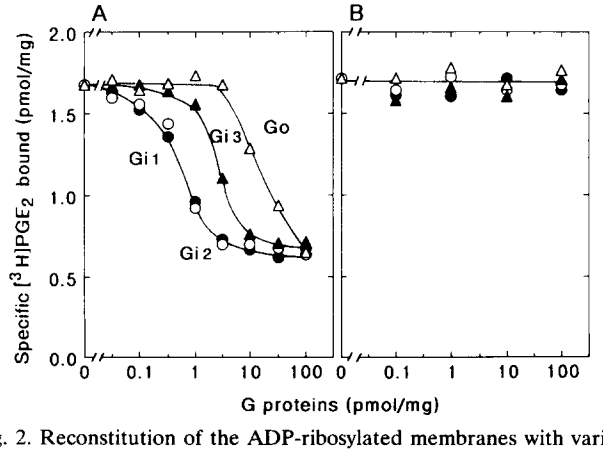

Fig. 2. Reconstitution of the ADP-ribosylated membranes with various G proteins. Various amounts of Gi1 (●), Gi2 (○), Gi3 (▲) and Go (△) were reconstituted into the ADP-ribosylated membranes of CHO cells as described under Materials and Methods. Reconstituted membranes were assayed for $[^3H]PGE_2$ binding activity in the presence (B) or absence (A) of 100 μM GTPγS. The values shown are the means for triplicate experiments, which varied by less than 5%.

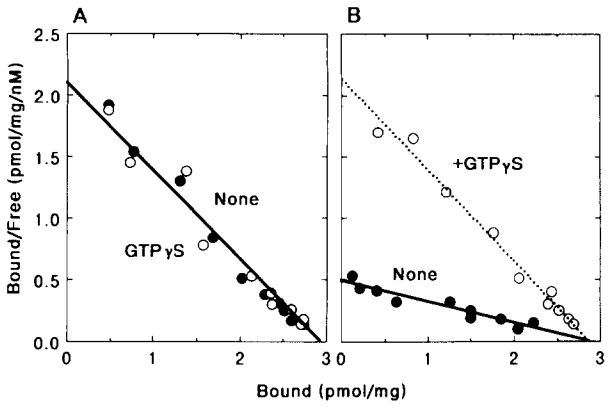

Fig. 3. Effect of GTPγS on the equilibrium binding of $[^3H]PGE_2$ to the ADP-ribosylated membranes reconstituted or not reconstituted with Gi2. Gi2 (10 pmol/mg of the membrane protein) was reconstituted into the ADP-ribosylated membranes as described under Materials and Methods. The membranes reconstituted (B) or not reconstituted (A) with Gi2 were incubated for 60 min at 30°C with increasing concentrations of $[^3H]PGE_2$ (0.25 to 20 nM) in the presence (○) or absence (●) of 100 μM GTPγS. Specifically bound $[^3H]PGE_2$ was determined as described under Materials and Methods. The Scatchard plot was transformed from the value of specific $[^3H]PGE_2$ binding.

These results demonstrate that the coupling of EP_3 receptor with G proteins reduces the ligand binding activity and GTPγS removes the reduction. To further elucidate whether the inhibitory effect of G proteins on the specific PGE_2 binding was due to a change in the number of binding site (B_{max}) or in the binding affinity (K_d), we carried out saturation experiments. Fig. 3 shows the results of Scatchard analysis of the specific PGE_2 binding in the presence or absence of GTPγS to the ADP-ribosylated membranes reconstituted or not reconstituted with Gi2. The K_d (5.7 nM) of EP_3 receptor in the membranes reconstituted with Gi2 was 4-fold higher than that (1.4 nM) in the membranes not reconstituted with Gi2, but B_{max} of EP_3 receptor in both membranes was the same value (2.9 pmol/mg). GTPγS decreased the K_d in the membranes reconstituted with Gi2 without any change in B_{max}, the decreased K_d (1.3 nM) being the same value as that in the membranes not reconstituted with Gi2. On the other hand, GTPγS had no effect on both K_d and B_{max} of EP_3 receptor in the membranes not reconstituted with Gi2. Therefore, the reduction of PGE_2 binding by G proteins is due to decrease in the binding affinity.

We further examined kinetic properties for the PGE_2 binding to the membranes. Fig. 4A shows the time courses of the specific PGE_2 binding to the membranes. The specific PGE_2 binding to the ADP-ribosylated membranes not reconstituted with Gi2 reached equilibrium within 50 min at 30°C, the half-time for the binding being about 5 min. GTPγS did not affect the time course of the binding to the membranes not

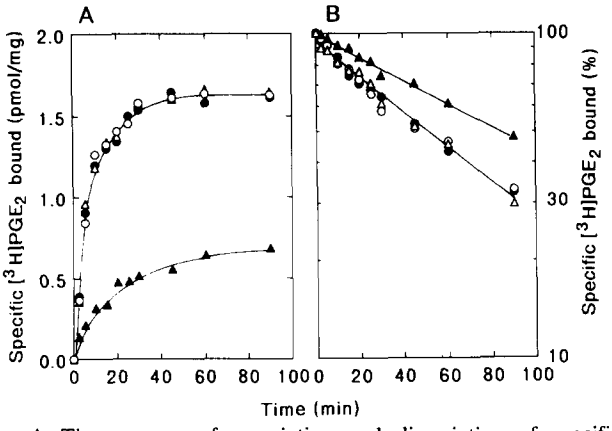

Fig. 4. Time-courses of association and dissociation of specific $[^3H]PGE_2$ binding to the ADP-ribosylated membranes reconstituted or not reconstituted with Gi2. Gi2 (10 pmol/mg of the membrane protein) was reconstituted into the ADP-ribosylated membranes as described under Materials and Methods. (A) The membranes reconstituted (▲, △) or not reconstituted (●, ○) with Gi2 were incubated at 30°C with 2 nM $[^3H]PGE_2$ in the presence (○, △) or absence (●, ▲) of 100 μM GTPγS. The amounts of specifically bound $[^3H]PGE_2$ were determined at various time intervals. (B) After the membranes reconstituted (▲, △) or not reconstituted (●, ○) with Gi2 had been labeled with 2 nM $[^3H]PGE_2$ for 1 h, they were washed and further incubated at 30°C in the presence (○, △) or absence (●, ▲) of 100 μM GTPγS. The amounts of bound $[^3H]PGE_2$ were determined at various time intervals as described under Materials and Methods. Nonspecifically bound $[^3H]PGE_2$ was determined under the same conditions. All values were corrected for nonspecifically bound $[^3H]PGE_2$ and represent the means of triplicate experiments, which varied by less than 5%.

reconstituted with Gi2. In contrast, the PGE_2 binding to the membranes reconstituted with Gi2 showed the lower equilibrium level and the longer half-time than those in the membranes not reconstituted with Gi2, but GTPγS increased the equilibrium level and decreased the half-time, the levels being the same values as those in the membranes not reconstituted with Gi2. On the other hand, the dissociation of the specifically bound PGE_2 from the membranes reconstituted with Gi2 was slower than that from the membranes not reconstituted with Gi2 (Fig. 4B). Whereas GTPγS did not affect the rate of dissociation of the bound PGE_2 from the membranes not reconstituted with Gi2, GTPγS promoted it from the membranes reconstituted with Gi2, the rate of dissociation being the same level as that in the membranes not reconstituted with Gi2. These results indicate that Gi2 decreases both the association and the dissociation rates of the ligand binding to EP_3 receptor, but GTPγS accelerates turnover of the ligand interaction with the receptor.

To reveal the physical association of EP_3 receptor with Gi2, $[^3H]PGE_2$-bound receptors solubilized from the membranes were analyzed by gel filtration on a Superose 12 column. As shown in Fig. 5A, $[^3H]PGE_2$-bound proteins solubilized from the membranes not reconstituted with Gi2 were eluted at the position of M_r = approx. 60 kDa in addition to being found in the void volume without regard to the presence of GTPγS. In marked contrast, $[^3H]PGE_2$-bound proteins solubilized from the membranes reconstituted with Gi2 were eluted at the position of M_r = approx. 130 kDa (Fig. 5B). However, $[^3H]PGE_2$-bound proteins in the presence of GTPγS solubilized from the membranes reconstituted with Gi2 were mainly eluted at the position of M_r = approx. 60 kDa corresponding to that of the proteins solubilized from the membranes not reconstituted with Gi2. Since the molecular weight of EP_3 receptor deduced from its amino acid sequence is 40077 and EP_3 receptor has two potential *N*-glycosylation sites, possibly showing higher molecular weight than 40077, the $[^3H]PGE_2$-bound proteins eluted at the position of M_r = approx. 60 kDa and about 130

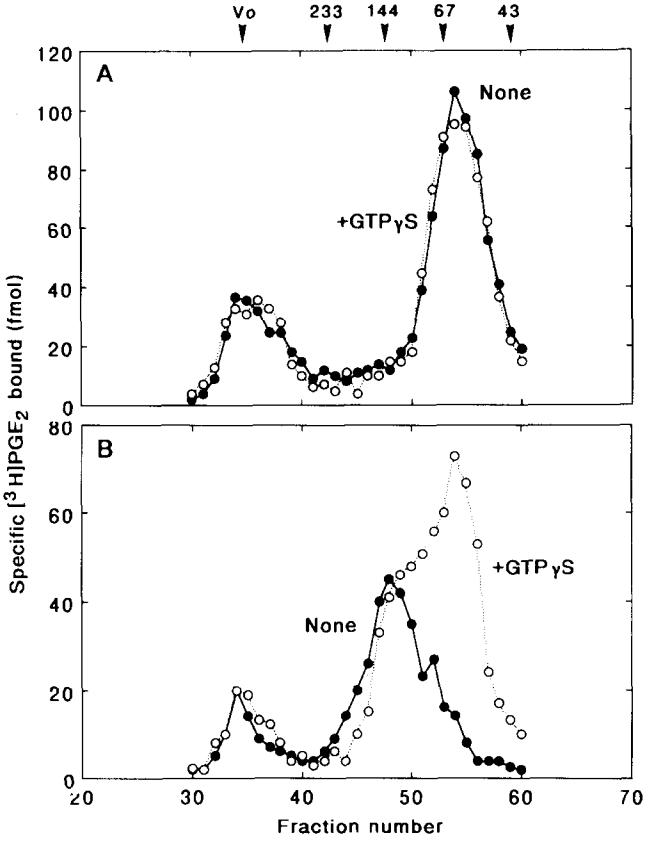

Fig. 5. Gel filtration of $[^3H]PGE_2$-bound proteins solubilized from the ADP-ribosylated membranes reconstituted or not reconstituted with Gi2. Gi2 (10 pmol/mg of the membrane protein) was reconstituted into the ADP-ribosylated membranes as described under Materials and Methods. After the membranes reconstituted (B) or not reconstituted (A) with Gi2 had been labeled with 2 nM $[^3H]PGE_2$ for 1 h in the presence (○) or absence (●) 100 μM GTPγS, they were solubilized with 20 mM CHAPS. The solubilized proteins were chromatographed on a Superose 12 HR 10/30 column as described under Materials and Methods. The radioactivity of $[^3H]PGE_2$ in each fraction was determined. Arrows indicate the retention volumes of known protein standards. The results shown are representative of three independent experiments.

kDa are supposed to be a monomeric EP_3 receptor and the complex of EP_3 receptor and Gi2, respectively.

GDP release from G proteins and Gi activity in the reconstituted membranes

The functional interactions occurring between EP_3 receptor and G proteins can be monitored by measuring the agonist-induced activation of G proteins. For this purpose in our experimental condition, GTPase activity and GTPγS binding activity assays are inappropriate, since endogenous GTPase activity or GTPγS binding activity in the membranes can not be inhibited by PT treatment. Therefore, we measured GDP release from [^{3}H]GDP-prelabeled G proteins reconstituted into the ADP-ribosylated membranes, which specifically reflects the activity of exogenously added G proteins. As shown in Fig. 6, PGE_2 induced a dose-dependent stimulation of GDP release over the basal release from Gi1 and Gi2, the half-maximal stimulation being occurred at about 0.2 nM, and produced the maximal release of GDP (50%) at 10 nM. PGE_2-stimulated GDP release was also observed in the reconstitution with Gi3 and Go, but the maximal release of GDP from Gi3 and Go was lower than that of Gi1 and Gi2 in that order. The rank order of PGE_2-stimulated GDP release from G proteins agreed well with those determined for the abilities of the G proteins to suppress the PGE_2 binding to the reconstituted membranes (Fig. 2).

Furthermore, we examined the inhibitory effect of PGE_2 on forskolin-stimulated adenylate cyclase activity in the G-protein-reconstituted membranes. In the control membranes, PGE_2 markedly attenuated forskolin-stimulated adenylate cyclase activity by about 50%, and this attenuation was not observed in the ADP-ribosylated membranes (data not shown). Addition of various amounts of Gi1, Gi2 and Gi3 restored the inhibitory effect of PGE_2 on forskolin-stimulated adenylate cyclase activity in a dose-dependent manner (EC_{50} values were determined to be 0.5, 0.5, and 20 pmol/mg of the membrane protein, respectively), but Go had no ability to restore the action of PGE_2 up to 100 pmol/mg (Fig. 7). Addition of these G proteins themselves did not affect the stimulation of the adenylate cyclase activity by forskolin (data not shown). Furthermore, when these G proteins were first ADP-ribosylated by PT and then reconstituted into ADP-ribosylated membranes, it was unable to restore the inhibitory action of PGE_2 (data not shown). These results demonstrate that EP_3 receptor is functionally coupled to these G proteins with a selectivity order of Gi1 = Gi2 > Gi3, and modulates the adenylate cyclase activity via the G proteins.

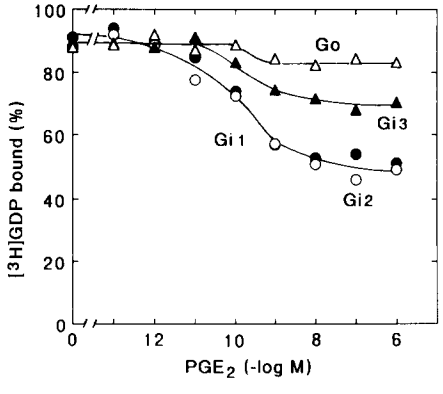

Fig. 6. Effect of PGE_2 on GDP release from various G-proteins reconstituted into the ADP-ribosylated membranes. [^{3}H]GDP-labeled Gi1 (●), Gi2 (○), Gi3 (▲) and Go (△) (10 pmol/mg of the membrane protein) were reconstituted into the ADP-ribosylated membranes as described under Materials and Methods. [^{3}H]GDP release from the indicated G proteins was measured in a 10-min incubation of the reconstituted membranes with increasing concentrations of PGE_2. GDP release was expressed as a percentage of total [^{3}H]GDP bound to each G protein reconstituted into the membranes and represents the mean of triplicate experiments, which varied by less than 5%. The dpm of total [^{3}H]GDP bound to each G protein was 8726±125 for Gi1, 7892±96 for Gi2, 9627±78 for Gi3, and 10296±148 for Go.

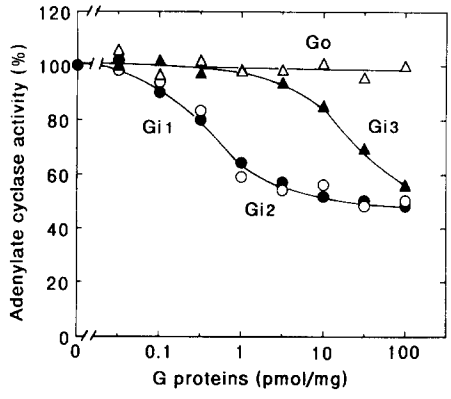

Fig. 7. Inhibitory effect of PGE_2 on the adenylate cyclase activity in the ADP-ribosylated membranes reconstituted with various G proteins. Various amounts of Gi1 (●), Gi2 (○), Gi3 (▲) and Go (△) were reconstituted into the ADP-ribosylated membranes as described under Materials and Methods. The reconstituted membranes were assayed for adenylate cyclase activity in the presence of 1 μM forskolin with or without 0.1 μM PGE_2. The values are expressed as a percentage of the control (158±13 pmol/min per mg) obtained with the ADP-ribosylated membranes in the absence of PGE_2 and represent the means of triplicate experiments, which varied by less than 5%.

Discussion

Recently, we have cloned a cDNA for the mouse PGE_2 receptor EP_3 subtype, which belongs to the family of G-protein-coupled rhodopsin-type receptors, and couples to inhibition of adenylate cyclase [17], suggesting the association of EP_3 receptor with Gi. In this study, we have characterized the interaction of EP_3

receptor with G proteins and discrimination of G proteins in a reconstituted system.

In binding studies on various hormone receptors that couple to G proteins, it has been observed that GTP decreases the receptor affinities for hormone agonists. This effect reflects the fact that GTP causes the dissociation of the G proteins from the receptors, leading to the transition of the receptors to a form that displays low-affinity agonist binding [13–15]. Therefore, generally receptors associated with G proteins show high-affinity binding of the hormones but receptors not associated with G proteins show low-affinity binding. On the other hand, in hamster adipocytes [16] and canine renal outer medulla [12], GTP unusually increased the PGE_2 binding affinities of the PGE_2 receptors, which are supposed to be EP_3 subtype. Here, we examined the mechanism of this enhancement by GTP in CHO cell membranes stably expressing EP_3 receptor reconstituted with various PT-sensitive G proteins. Specific binding of PGE_2 to the membranes of control CHO cells was enhanced by GTPγS, and PT treatment also increased the PGE_2 binding which reached the level of GTPγS-stimulated PGE_2 binding to the control cell membranes (Fig. 1C). When PT-sensitive G proteins were reconstituted into the ADP-ribosylated CHO cell membranes, the level of PGE_2 binding declined to that on the control cell membranes (Fig. 2). These results indicate that PGE_2 binding activity of G-protein-associated EP_3 receptor is lower than that of EP_3 receptor not associated with G proteins. From the Scatchard plot analysis (Fig. 3), the low level of PGE_2 binding activity of G protein-associated EP_3 receptor is due to decrease in the binding affinity but not due to decrease in the receptor number, and GTPγS increases the binding affinity by dissociation of G proteins from EP_3 receptor. Therefore, the complex form of EP_3 receptor with G proteins has low affinity for PGE_2 but the receptor not associated with G-proteins has high affinity. On the other hand, as expected from studies of other hormone receptor systems [15], it is clear in EP_3 receptor that the addition of agonist accelerates the dissociation of prebound GDP (Fig. 6) and a stable ternary complex, agonist, receptor and G protein, only occurs in the absence of guanine nucleotides (Fig. 4), and that the addition of GTP induces dissociation of receptor-G-protein complex (Fig. 5). Therefore, the ternary complex represents a transition state in the guanine nucleotide exchange reaction, and the function of the agonist is to stabilize this transition state and to promote the exchange rate, which is a critical step for receptor-mediated signal transduction. Although previous works of most receptors had emphasized the idea that the high-affinity state of receptors is the active conformation while the low-affinity state represents uncoupled inactivated receptor, this notion cannot apply to EP_3 receptor and is not generally accepted. Considering the high efficiency of agonist for activation of G proteins in EP_3 receptor-G-protein reconstitution system (Fig. 6), EP_3 receptor having high affinity for agonist in G-protein-free form would modulate the efficiency of EP_3 receptor for G-protein activation.

We further examined specificity of G proteins coupled to EP_3 receptor and whether the interaction of the receptor with G proteins is functional. Previous studies of receptor-G-protein coupling have revealed some specificity of coupling to G proteins. For example, the β-adrenergic receptor couples to Gs more efficiently than either Gi, Go or Gt, as evidenced by agonist stimulation of GTPase activity [26]. On the other hand, receptors coupled to inhibition of adenylate cyclase activity showed the following preference between the different PT-sensitive G proteins: α_2-adrenergic receptor, Gi3 > Gi1 $\geqq$ Gi2 > Go [27]; D_2 dopaminergic receptor, Gi2 > Gi1 = Gi3 $\gg$ Go [28]; A_1 adenosine receptor, Gi3 $\gg$ Go = Gi1 = Gi2 [29]. From the specificity of G proteins for their ability to decrease PGE_2 binding activity (Fig. 2), EP_3 receptor can couple to multiple PT-sensitive G proteins, in the order of Gi1 = Gi2 > Gi3 > Go. It has been shown that receptor-mediated activation of G proteins is the direct outcome of receptor-induced dissociation of bound GDP from the G proteins [30]. Judging from the intensity of PGE_2-stimulated dissociation of bound GDP from G proteins, EP_3 receptor functionally interacted with Gi1, Gi2, Gi3 and Go (Fig. 6), and this rank order of PGE_2-stimulated GDP release from G proteins was comparable with that for G-protein-mediated reduction of PGE_2 binding (Fig. 2). Furthermore, PGE_2 inhibited the adenylate cyclase activity through coupling of EP_3 receptor with multiple Gi also in the order of Gi1 = Gi2 > Gi3, but Go could not serve the inhibitory action of PGE_2 (Fig. 7). Various α subunits of Gi by themselves have been shown to inhibit adenylate cyclase activity in the order of Gi1 > Gi2 > Gi3, but Goα had no ability to inhibit it even at the high concentrations [31]. Considering the finding that Go could not serve the inhibitory action of PGE_2, inhibition of adenylate cyclase activity by EP_3 receptor is assumed to be mediated by α subunits of Gi themselves but not by indirect action of dissociated $\beta\gamma$ subunits. In contrast to other Gi-coupled receptors, α_2-adrenergic, D_2 dopaminergic, and A_1 adenosine receptors, EP_3 receptor prefers Gi1 and Gi2 to Gi3 and Go, and the preference of Gi1 and Gi2 by EP_3 receptor for its coupling is appropriate to the strong ability of Gi1 and Gi2 to inhibit adenylate cyclase activity. The evidence that EP_3 receptor actually interacts with Gi1 or Gi2 has been shown by the observation that antibody against Gi1α and Gi2α selectively precipitated PGE_2 binding activity in canine renal medulla [32]. On the other hand, it is evident that EP_3

receptor can potentially activate Gi3 and even Go. It also seems clear that these G proteins may be interacting with other effector systems such as activation of K^+ channels and inhibition of Ca^{2+} channels [33], suggesting that EP_3 receptor regulates these effector systems. Since the rank order of potencies of G proteins for the reduction of PGE_2 binding affinity agreed well with those for EP_3 receptor-mediated activation of G proteins and subsequent inhibition of adenylate cyclase activity, the interaction of EP_3 receptor with these G proteins, showing low-affinity ligand binding, is functional and EP_3 receptor exerts its inhibitory action for adenylate cyclase through this interaction with the G proteins.

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