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Functional Reconstitution of Platelet Thromboxane A_2 Receptors with G_q and G_{i2} in Phospholipid Vesicles

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SUMMARY

The partially purified thromboxane (TX) A_2 receptor was reconstituted with two species of purified heterotrimeric G proteins, G_q and G_{i2}, in phospholipid vesicles. The receptors reconstituted with G_q and G_{i2} showed a single class of [^{3}H]S-145 binding, with K_d values of 9.6 ± 0.7 and 12.1 ± 1.0 nM, respectively; binding was displaced by GR32191, 9,11-epithio-11,12-methano-thromboxane A_2 (STA_2), and U46619, with almost identical K_i values for each compound in the two types of reconstituted vesicles. When the receptor and G_q were reconstituted, the agonist STA_2 stimulated guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding. This stimulation was half-maximal at 80 nM and reached a plateau at 1 μM. At 10 μM STA_2 stimulated the initial rate by 20–30-fold, compared with the basal rate. The stimulation of guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding to G_{i2} by the agonist-liganded receptor was seen in the presence of GDP. Under these conditions, 10 μM STA_2 stimulated the initial rate by 1.5–2-fold, compared with the basal rate. This effect was half-maximal at 150 nM and reached a plateau at 1 μM. The agonist-liganded receptor also stimulated the GTPase activities of the reconstituted G proteins. The steady state rates of STA_2-stimulated [^{32}P]P_i release from [γ-^{32}P]GTP were 2.21/min·receptor and 0.87/min·receptor in the G_q- and G_{i2}-reconstituted vesicles, respectively, and the k_{cat} values of G_q and G_{i2} in the presence of STA_2 were 0.87 ± 0.21 min^{-1} and 2.41 ± 0.12 min^{-1}, respectively. These results clearly show that the TXA_2 receptor functionally couples to both G_q and G_{i2}. Consistent with this finding, STA_2, by acting on the TXA_2 receptor in intact platelets, inhibited prostaglandin I_2-induced cAMP elevation.

TXA_2, a major cyclooxygenase metabolite of arachidonate in platelets, is a potent stimulator of platelets and a constrictor of vascular and airway smooth muscles (1). We have purified TXA_2 receptors from human platelets (2) and cloned cDNAs for human and mouse TXA_2 receptors (3, 4). Those studies revealed that these receptors belong to the family of rhodopsin-type G protein-coupled receptors. However, the precise mechanisms of their signal transduction have not been fully elucidated. TXA_2 induces phosphatidylinositol breakdown through the activation of PLC in a variety of cells, including platelets, and this pathway is known to be resistant to pertussis toxin treatment (5, 6). Recently, two closely related G proteins, G_q and G_{11}, have been identified as pertussis toxin-insensitive activators of PLC-β_1 (7). Shenker *et al.* (8) reported that an antibody against the $G_{q/11}$[1] family of G proteins partially inhibited U46619 (a TXA_2 agonist)-induced increases in the GTPase activity of platelet membranes. Knezevic *et al.* (9) reported that the TXA_2 receptor was co-purified with a G protein of the $G_{q/11}$ family from human platelets. Although these results suggest the involvement of the $G_{q/11}$ family of G proteins in signal transduction for the platelet TXA_2 receptor, direct coupling of the receptor to $G_{q/11}$ has not been shown and the identity of the coupling $G_{q/11}$ protein(s) has not been elucidated. TXA_2 also stimulates arachidonate release from platelets, and this action is reported to be mediated by a G protein distinct from that required for PLC activation (10). Furthermore, although it is well established that thrombin and α-adrenergic receptor agonists inhibit platelet adenylate cyclase apparently via the inhibitory G protein G_i (11, 12), there are conflicting reports concerning the inhibition of platelet adenylate cyclase by the TXA_2 receptor (for review, see Ref. 13). It is also known that TXA_2 can stimulate the growth of aortic smooth muscle cells (14), HeLa cells (15), and peripheral T cells (16), but the identity of the G protein coupling to the receptor in these cells is not known. TXA_2 also induces Ca^{2+} influx, which is regulated by G proteins in some situations (13, 17).

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[1] We refer to mixtures of G_q and G_{11} as $G_{q/11}$ and their α subunits as $\alpha_{q/11}$. The two α subunits are closely related and form a distinct subfamily (7). We also refer to the α subunits of G_{i1}, G_{i2}, and G_{i3} as α_{i1}, α_{i2}, and α_{i3}, respectively.

ABBREVIATIONS: TX, thromboxane; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; S-145, 5*Z*-7-(3-*endo*-phenylsulfonylaminobicyclo[2.2.1]hept-2-*exo*-yl)heptenoic acid; GR32191, [1R-[1α(*Z*),2β,3β,5α]]-(+)-7-[5-[[(1,1′-biphenyl)-4-yl]methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid hydrochloride; U46619, 15(*S*)-hydroxy-11,9-epoxymethano-prosta-5*Z*,13*E*-dienoic acid; STA_2, 9,11-epithio-11,12-methano-thromboxane A_2; PLC, phospholipase C; PLA_2, phospholipase A_2; App(NH)p, adenosine 5′-(β,γ-imido)triphosphate; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

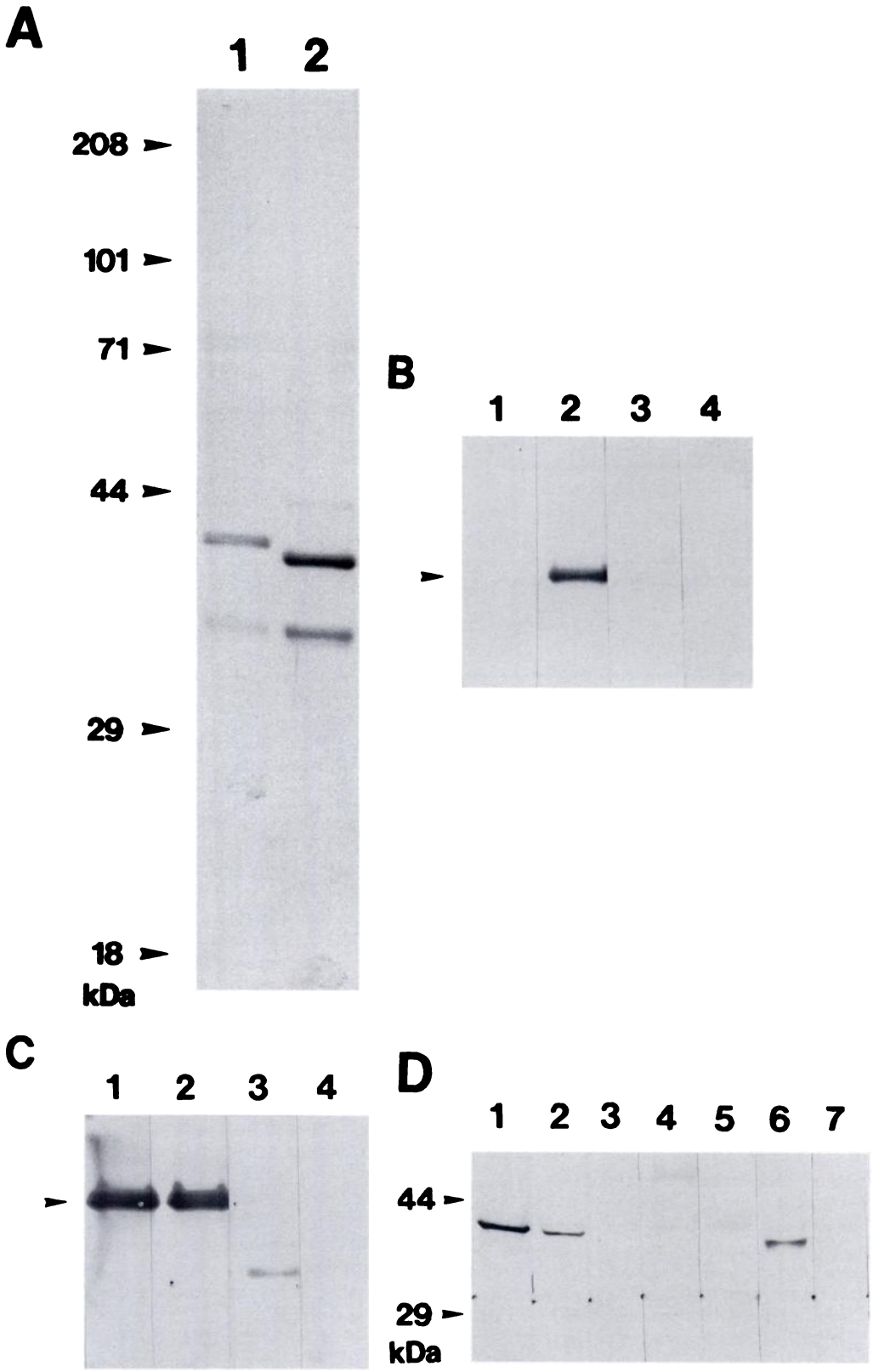

Fig. 1. A, SDS-polyacrylamide gel electrophoresis and silver staining of the purified G proteins. Final preparations were dissolved in Laemmli buffer and subjected to SDS-polyacrylamide gel electrophoresis in 10% gels. Proteins were stained with silver reagent. *Lane 1*, $G_{q/11}$ protein (0.2 μg); *lane 2*, G_i protein (0.6 μg). *Numbers to the left*, positions of the molecular weight standards. B, Identification of the purified G_i as G_{i2}. After SDS-polyacrylamide gel electrophoresis, G_i was transferred to an Immobilon membrane and probed with antiserum specific for α_{i1} (*lane 1*), α_{i2} (*lane 2*), or α_{i3} (*lane 3*) or with control serum (*lane 4*), as described in Experimental Procedures. *Arrowhead*, position of the 40-kDa α_i subunit. C, Identification of the purified $G_{q/11}$ as G_q. After SDS-polyacrylamide gel electrophoresis, $G_{q/11}$ was transferred to an Immobilon membrane and probed with the anti-QL peptide antiserum (*lane 1*), the antiserum specific for α_q (E973) (*lane 2*), the antiserum specific for α_{11} (E976) (*lane 3*), or control serum (*lane 4*),

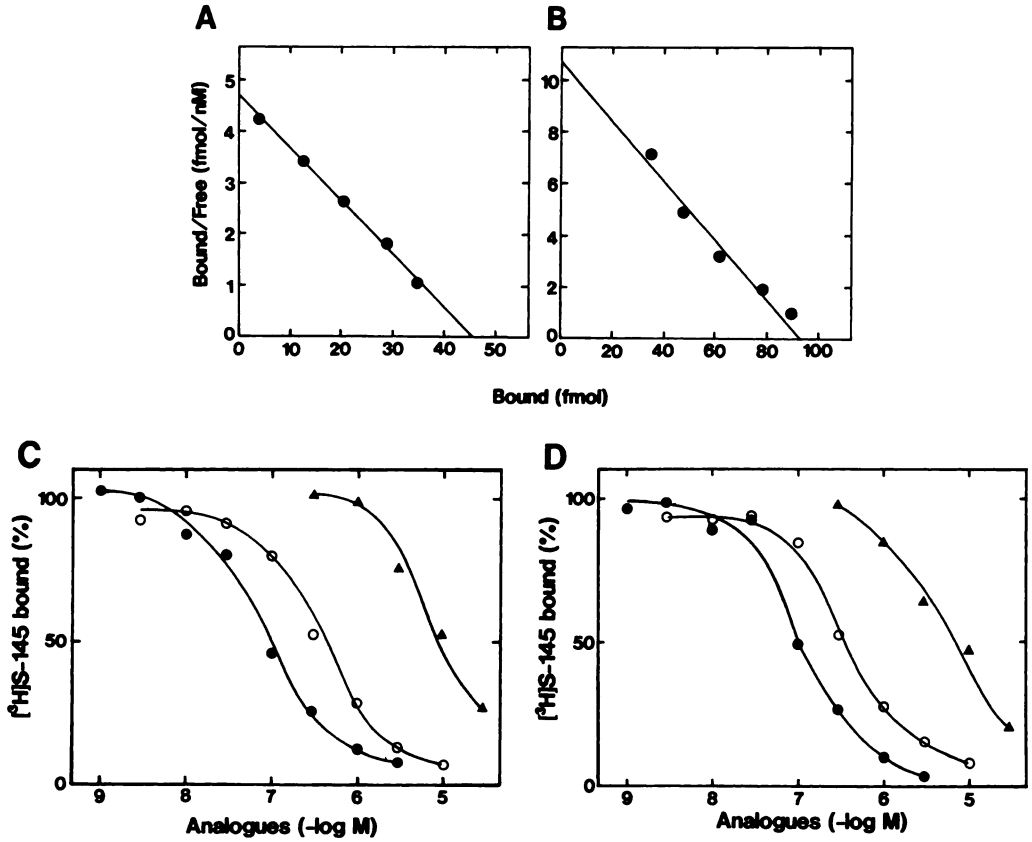

Fig. 2. A and B, Scatchard plots of [^{3}H]S-145 binding to the TXA_2 receptor in vesicles reconstituted with G_q (A) or G_{i2} (B). The reconstituted vesicles were incubated with various concentrations of [^{3}H]S-145 and the bound radioactivity was measured as described in Experimental Procedures. C and D, Displacement by TXA_2 agonists and antagonist of the specific binding of [^{3}H]S-145 in vesicles reconstituted with G_q (C) or G_{i2} (D). Unlabeled TXA_2 analogues were added to the binding assay mixture at the indicated concentrations, and specific [^{3}H]S-145 binding was determined as described in Experimental Procedures (mean values, four experiments). ●, GR32191; ○, STA_2; ▲, U46619.

The aforementioned diverse effects of TXA_2 may originate from the diversity of the G proteins coupling to the TXA_2 receptor. To test this possibility, we utilized a reconstitution system in which purified receptors activate the coupling G proteins selectively (18, 19). In this study, we purified two G proteins, G_q and G_{i2}, from human platelet membranes and found that they couple effectively and functionally to the TXA_2 receptor. This may explain, at least in part, the multiplicity of signal transduction through the TXA_2 receptor.

Experimental Procedures

Materials. [^{3}H]S-145 (24.5 Ci/mmol) and S-145 were gifts from Shionogi Research Laboratories (Osaka, Japan). [^{35}S]GTPγS (1244 Ci/mmol), [α-^{32}P]NAD (500 Ci/mmol), and [γ-^{32}P]GTP (6000 Ci/mmol) were obtained from DuPont-New England Nuclear. GDP, NAD, App(NH)p, phosphocreatinine, creatine phosphokinase, 3-isobutyl-1-methylxanthine, and cholesteryl hemisuccinate were purchased from Sigma Chemical Co. (St. Louis, MO). STA_2 was a gift from Ono Pharmaceuticals (Osaka, Japan). U46619 was purchased from Upjohn (Kalamazoo, MI). GR32191 was kindly supplied by Dr. R. A. Coleman (Glaxo Research Ltd., Ware, England). Tween-20 was obtained from Nakarai Tesque, Inc. (Kyoto, Japan). All other chemicals used were of reagent grade. Sources of other materials used were described previously (2).

Antibodies and immunoblotting. Antisera against G_q (E973) and G_{11} (E976) (20) were kindly provided by Dr. J. H. Exton, Vanderbilt University, and antisera against G_{i1}, G_{i2}, and G_{i3} (21) by Dr. Y. Kanaho, The Tokyo Institute of Technology. Each of these anti-G_i antisera could detect 0.1–0.25 μg of the respective protein on Western blotting. Purified G proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and then transferred to Immobilon membranes (Millipore). After the membranes were blocked with 3% bovine serum albumin in Tris-buffered saline for >1 hr, they were incubated for 1 hr with 1/500 dilutions of the antisera in Tris-buffered saline with 0.05% Tween-20. The bound antibodies were detected with an ABC kit (Vector Laboratories, Burlingame, CA). All procedures for immunoblotting were done at room temperature. Antisera against the common carboxyl-terminal decapeptide of α_q and α_{11} (QL peptide) were raised according to the method of Shenker *et al.* (8). When platelet membranes were analyzed by SDS-polyacrylamide gel electrophoresis and Western blot analysis using the anti-QL antisera, a single protein band of 42 kDa was detected.

Purification of the TXA_2 receptor from platelet membranes. The TXA_2 receptor was solubilized with 10 mM CHAPS and partially purified (about 1500-fold) by chromatography on Affi-Gel-S-145 and

as described in Experimental Procedures. *Arrowhead*, position of the 42-kDa $\alpha_{q/11}$ subunit. D, Identification of $G_{q/11}$ and G_i families of G proteins in platelet membrane. Platelet membranes (35 μg of protein) were dissolved in Laemmli buffer and subjected to SDS-polyacrylamide gel electrophoresis. After the proteins were transferred to an Immobilon membrane, they were probed with anti-QL peptide antiserum (*lane 1*), the antiserum specific for α_q (E973) (*lane 2*), the antiserum specific for α_{11} (E976) (*lane 3*), control serum (*lane 4*), the antiserum specific for α_{i1} (*lane 5*), the antiserum specific for α_{i2} (*lane 6*), or the antiserum specific for α_{i3} (*lane 7*), as described in Experimental Procedures. *Numbers to the left*, positions of molecular weight standards.

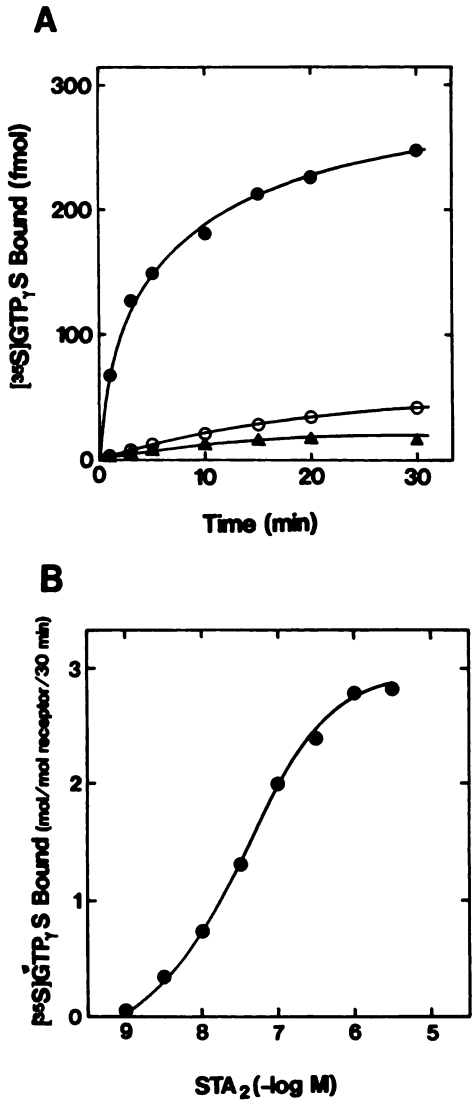

Fig. 3. A, Time course of [^{35}S]GTPγS binding to vesicles reconstituted with the TXA_2 receptor and G_q. The receptor and G_q were reconstituted in lipid vesicles and [^{35}S]GTPγS binding to the vesicles was assayed at the indicated times, in the presence of either 10 μM STA_2 (●), 1 μM S-145 (▲), or vehicle (○), as described in Experimental Procedures. The vesicle contained 38 fmol of receptor. B, Concentration-response curve for STA_2 effects on [^{35}S]GTPγS binding to vesicles reconstituted with the TXA_2 receptor and G_q. Vesicles reconstituted with the TXA_2 receptor and G_q were incubated with 7 nM [^{35}S]GTPγS and various concentrations of STA_2 for 30 min at 30°. The bound [^{35}S]GTPγS was assayed as described in Experimental Procedures (mean values, four experiments).

wheat germ agglutinin-agarose columns (2). This preparation was 19% pure, contained negligible amounts of G proteins, as assessed by [^{35}S]GTPγS binding, and alone did not increase binding when stimulated with STA_2 in a reconstituted system.

Purification of G_i. All purification procedures were performed at 4°. Platelet membranes (5 g of protein) were prepared (2) and solubilized for 30 min with 10 mM CHAPS in 1000 ml of TEM buffer (20 mM Tris·HCl, pH 7.4, 1 mM EDTA, 3 mM $MgCl_2$, 1 mM DTT, 1 μM GDP, 10 units/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride, 1 mM benzamidine). The solubilized fraction (800 ml) was obtained by centrifugation at 100,000 × *g* for 1 hr and was applied to a DEAE-Sepharose column (2.6-cm i.d. × 16 cm, 80 ml) that had been preequilibrated with TEMC (TEM buffer containing 10 mM CHAPS). After the column was washed with TEMC, elution was performed with a linear gradient of 0–0.25 M NaCl in 800 ml of TEMC. G_i, identified

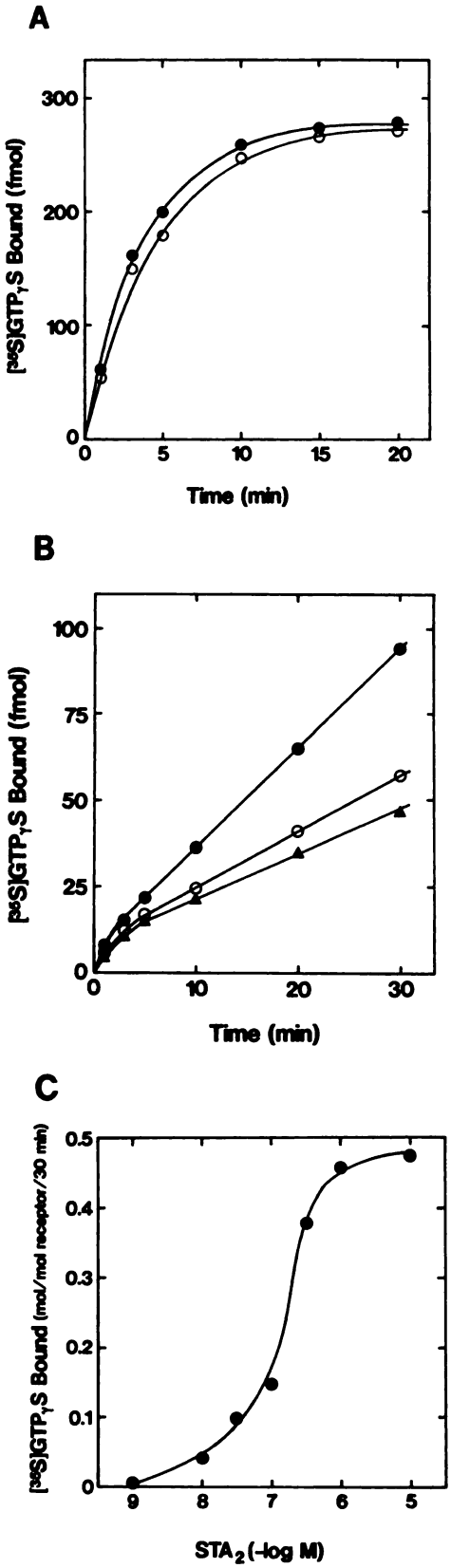

Fig. 4. A and B, Time course of [^{35}S]GTPγS binding to reconstituted vesicles containing the TXA_2 receptor and G_{i2} protein, in the absence (A) or presence (B) of 1 μM GDP. The receptor and G_{i2} were reconstituted in lipid vesicles and [^{35}S]GTPγS binding to the vesicles was assayed at the indicated times, in the presence of 10 μM STA_2 (●), 1 μM S-145 (▲), or vehicle (○), as described in Experimental Procedures. The vesicles

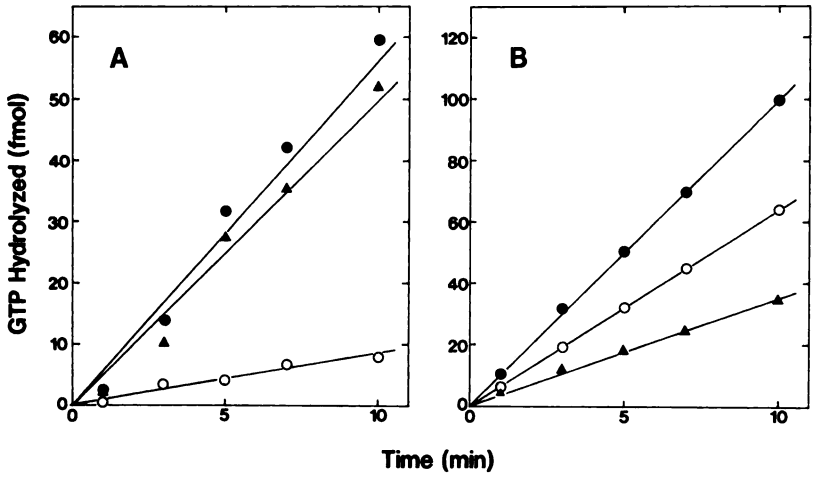

Fig. 5. Time course of STA_2-stimulated [γ-^{32}P]GTP hydrolysis in vesicles reconstituted with the TXA_2 receptor and G_q (A) or G_{i2} (B). The receptor and either G_q or G_{i2} were reconstituted in lipid vesicles and incubated with 0.1 μM [γ-^{32}P]GTP in the presence of 10 μM STA_2 (●) or 10 μM S-145 (○). At the indicated times, liberated [^{32}P]P_i was measured as described in Experimental Procedures. GTP hydrolysis in the presence of S-145 was subtracted from that seen in the presence of STA_2 and the differences are also shown (▲).

by [^{35}S]GTPγS binding and [^{32}P]ADP-ribosylation by pertussis toxin, was eluted in a single peak at 0.17 M NaCl. The peak fraction, concentrated to 8 ml, was applied to a Sephacryl S-300 column (2.6-cm i.d. × 48 cm, 255 ml) that had been preequilibrated with TEMC containing 0.5 M NaCl and was eluted with the same buffer. The activity appeared in a single symmetrical peak with an elution volume of approximately 160 ml. This fraction (28 ml) was diluted to 140 ml with TEM containing 0.15 M NaCl and was applied to a phenyl-Sepharose column (1.6-cm i.d. × 10 cm, 20 ml) that had been preequilibrated with TEM containing 2 mM CHAPS and 0.25 M NaCl. After the column was washed with the preequilibration buffer, elution was performed with linear gradients of 2–12 mM CHAPS and 0.25–0 M NaCl in 200 ml of TEM. The activity was eluted in a single peak at 9.2 mM CHAPS and 0.03 M NaCl. The buffer of this fraction was changed to buffer A (5 mM sodium phosphate, pH 7.2, 0.5 mM EDTA, 1 mM DTT, 10 units/ml aprotinin, 1.5 mM $MgCl_2$, 10 mM CHAPS, 1 μM GDP) using a PD-10 column (Pharmacia). The fraction, concentrated to 500 μl, was injected onto an hydroxyapatite column (Tonnen) that was connected to a fast protein liquid chromatography system (Pharmacia LKB Biotechnology) and that had been preequilibrated with buffer A. After the column was washed with 10 ml of buffer A, elution was carried out with a linear gradient of 5–150 mM sodium phosphate, at a flow rate of 0.5 ml/min (total volume, 30 ml). The activity was eluted in a single peak at 80 mM sodium phosphate. After the buffer was changed to TEMC, the fraction was injected onto a Mono Q column that had been preequilibrated with TEMC. Elution was performed with a linear gradient of 0–0.3 M NaCl in TEMC, at a flow rate of 0.5 ml/min (total volume, 30 ml). The activity was eluted at 0.22 M NaCl in a single symmetrical peak. To remove small amounts of contaminating G_q, Mono Q column chromatography was repeated three times.

Purification of G_q. All of the purification procedures were performed at 4°. Platelet membranes (1 g of protein) were solubilized for 30 min with 10 mM CHAPS in 200 ml of TEG buffer (TEM buffer containing 10 μM GDP). The anti-QL antiserum (2 ml) was then added to the solubilized fraction and incubated for 1 hr. Protein A-Sepharose (2 ml, wet volume) was added and the mixture was incubated for another 1 hr, with rocking. After the incubation, the Sepharose beads were collected by centrifugation at 190 × *g* for 10 min and were packed into the column. The column was washed successively with 100 ml of TEGC (TEG containing 10 mM CHAPS) and 100 ml of TEGCS (TEGC containing 160 mM NaCl). $G_{q/11}$ was eluted from the beads, after a 20-hr incubation, with 100 μM QL peptide in 10 ml of TEGCS. After the buffer was changed to TEGC, the eluate was concentrated and applied to a Mono Q column that had been preequilibrated with TEGC. The column was then washed with 10 ml of TEGC, and elution was performed with a linear gradient of 0–0.3 M NaCl in TEGC, at a flow rate of 0.5 ml/min (total volume, 30 ml). $G_{q/11}$, identified by immunoblotting as a protein reactive with anti-QL antiserum, was eluted in a single peak at 0.24 M NaCl. This fraction (2.5 ml) was concentrated and injected onto a TSK-Gel G3000SW column that had been preequilibrated with TEMC containing 0.5 M NaCl. Elution was performed with TEMC containing 0.5 M NaCl, at a flow rate of 0.5 ml/min. The activity was eluted in a single symmetrical peak, which overlapped exactly with a protein peak at an elution volume of approximately 17 ml.

Reconstitution of the TXA_2 receptor and G proteins. The reconstitution of the receptor and G proteins was performed according to the method of Haga *et al.* (22). Lipid was extracted from platelet membranes with chloroform/methanol. The TXA_2 receptor (usually 50–100 pmol in 100 μl of 20 mM Tris·HCl, pH 7.4, 10 mM CHAPS, 1 mM EDTA, 20% glycerol, 50 μg/ml phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.5 M KCl) was mixed with 250 μl of lipid mixture [350 μg of lipid extract from platelet membranes, 5 μg of cholesteryl hemisuccinate, and 10 mM CHAPS in REC (20 mM Tris·HCl, pH 7.4, 1 mM EDTA, 3 mM $MgCl_2$, 160 mM NaCl)]. Five microliters of 1 mM STA_2, 15 μl of 100 mM CHAPS, and 130 μl of water were then mixed with this receptor/lipid mixture and incubated for 20 min at 37°. A 200-μl aliquot of the mixture was applied to a Sephadex G-50 column (bed volume, 2 ml) that had been preequilibrated with REC. Elution was performed with REC and the first 600 μl of the eluate were discarded. The next 400 μl of eluate, containing the receptor/lipid vesicles, were collected. The receptor/lipid vesicles were mixed with G proteins (50–300 pmol of G_q or 50–350 pmol of G_i), and the concentration of CHAPS in this mixture was adjusted to 1–2 mM, in a total volume of 500 μl. After incubation for 1 hr at 4°, this mixture was diluted gradually with REC to 3 ml, to make receptor/G protein-reconstituted vesicles. The yield of the receptor, determined in a [^{3}H] S-145 binding assay, was 2–6%, and this was not different between G_q- and G_i-reconstituted vesicles. The receptor/G protein ratio was 1:5 to 1:20 in the G_i-reconstituted vesicles. This ratio was not determined for the G_q-reconstituted vesicles because without agonist stimulation [^{35}S] GTPγS did not bind to G_q.

Binding assays. [^{35}S]GTPγS binding was performed as described previously (23). Briefly, the reconstituted vesicles were incubated at 30° with 7 nM [^{35}S]GTPγS (1393 Ci/mmol) in 100 μl of TEM containing 100 mM NaCl and 0.1 mM DTT. The reaction was terminated at the indicated times by the addition of 2 ml of ice-cold washing buffer (20 mM Tris·HCl, pH 7.4, 1 mM EDTA), and the reaction mixture was

contained 35 fmol (A) or 49 fmol (B) of receptor. C, Concentration-response curve for STA_2 effects on [^{35}S]GTPγS binding to vesicles reconstituted with the TXA_2 receptor and G_{i2}. Vesicles reconstituted with the TXA_2 receptor and G_{i2} were incubated with 7 nM [^{35}S]GTPγS and various concentrations of STA_2 for 30 min at 30°. The bound [^{35}S]GTPγS was assayed as described in Experimental Procedures (mean values, three experiments).

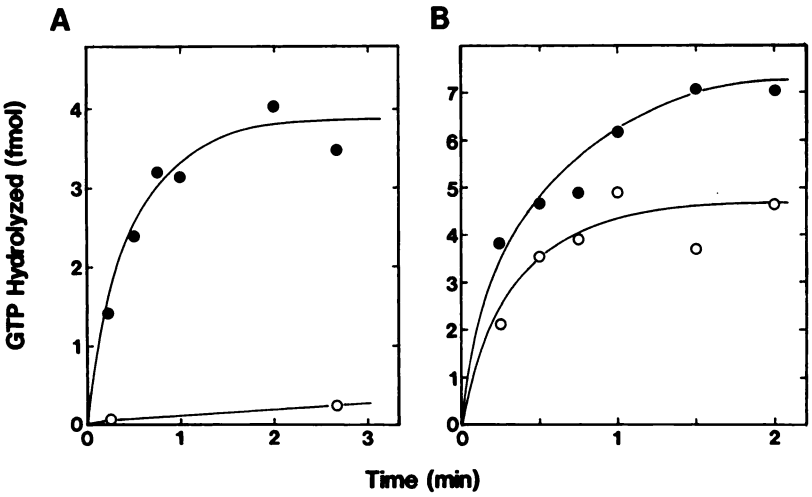

Fig. 6. Time course of the hydrolysis of bound [γ-^{32}P]GTP in vesicles reconstituted with the TXA_2 receptor and G_q (A) or G_{i2} (B). After the vesicles were preincubated with 0.1 μM [γ-^{32}P]GTP for 3 min, 1 mM unlabeled GTP was added and the liberated [^{32}P]P_i was measured at the indicated times, in the presence of 10 μM STA_2 (●) or 10 μM S-145 (○), as described in Experimental Procedures.

immediately filtered through a BA85 nitrocellulose membrane filter. The Scatchard analysis and the assays of displacement of [^{3}H]S-145 binding were performed as described previously (24).

GTPase assay. GTPase activities of reconstituted vesicles were determined according to the method of Cassel and Selinger (25), with slight modifications. The vesicles were incubated with the indicated concentrations of STA_2 or S-145 at 30° for 30 min. The vesicles were then incubated again at 30° with 0.1 μM [γ-^{32}P]GTP (200 Ci/mmol) in 50 μl of REC containing 100 mM NaCl, 0.1 mM DTT, 1 mM App(NH)p, 0.2 mM ATP, 5 mM phosphocreatine, and 50 units/ml creatine phosphokinase. At various times, the reaction was terminated by the addition of 950 μl of cold 5% (w/v) charcoal (Norit SX Plus; Wako Pure Chemical Industries, Osaka, Japan), in 20 mM sodium phosphate, pH 7.4. The mixture was centrifuged for 5 min at 10,000 × g at 4°, and the [^{32}P]P_i levels in 500-μl aliquots of supernatant were determined in liquid scintillator (Clear-sol I; Nakarai Tesque, Inc.). When the hydrolysis of bound [γ-^{32}P]GTP was measured, the vesicles were incubated with [γ-^{32}P]GTP for 3 min as described above, in a total volume of 400 μl. Unlabeled GTP at 1 mM was then added, and a 50-μl aliquot was taken and mixed with 950 μl of cold charcoal at the indicated times.

cAMP measurement. Washed platelet suspensions were prepared as reported previously (26), except that the platelets were suspended in HEPES buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, pH 7.4). After incubation of the platelets with various agents in the presence of 1 mM 3-isobutyl-1-methylxanthine for 10 min at 30°, the reaction was terminated with 6% trichloroacetic acid. cAMP contents were measured using a ^{125}I-cAMP radioimmunoassay kit (Amersham, England).

Results

Purification of G proteins. A G_i protein was purified as a [^{32}P]ADP-ribosylation substrate for pertussis toxin, with [^{35}S]GTPγS binding activity, and a $G_{q/11}$ protein was purified as a protein that was recognized by the anti-QL peptide antiserum. The purified proteins were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 1A). The G_i protein contained an α subunit of 40 kDa, and the $G_{q/11}$ protein contained an α subunit of 42 kDa. The 40-kDa α subunit was identified as $G_{i2\alpha}$ by Western blotting with subtype-specific antisera (Fig. 1B), which is consistent with the report that human platelet membranes contain large amounts of G_{i2} and little G_{i3} (11, 27). We also identified the 42-kDa α subunit as α_q of the $G_{q/11}$ family of G proteins (Fig. 1C). As reported previously (19), G_q protein, after being solubilized from platelet membranes, showed a low affinity for GTPγS; the K_d value of purified G_q for GTPγS was 960 nM. As shown in Fig. 1D, G_q and G_{i2} are the major proteins of the $G_{q/11}$ and G_i families of G proteins, respectively, in human platelets.

Functional coupling of the TXA_2 receptor to G_q and G_{i2}. The TXA_2 receptor and either G_q or G_{i2} were reconstituted in lipid vesicles as described in Experimental Procedures. The Scatchard plots of [^{3}H]S-145 binding to the receptor in the G_q- and G_{i2}-reconstituted vesicles are shown in Fig. 2. The plots yielded single straight lines, suggesting a single class of binding sites in both types of reconstituted vesicles. The K_d values were 9.6 ± 0.7 nM and 12.1 ± 1.0 nM in the G_q- and G_{i2}-reconstituted vesicles, respectively (mean ± standard error, four experiments). We also examined the affinities of TXA_2 agonists (STA_2 and U46619) and an antagonist (GR32191) for the receptor in the G_q- and G_{i2}-reconstituted vesicles, by displacement of [^{3}H]S-145 binding (Fig. 2). The K_i values were calculated from the equation $K_i = IC_{50}/(1 + [L]/K_d)$, in which IC_{50} is the concentration of a TXA_2 receptor analogue inhibiting specific radioligand binding by 50% and $[L]$ and K_d are the concentration and dissociation constant of the radioligand, respectively. There were no significant differences in the affinities of these ligands for the receptors reconstituted with G_q or G_i. The K_i values for GR32191, STA_2, and U46619 were 37.1 ± 2.6, 134 ± 10, and 4100 ± 390 nM in the G_q-reconstituted vesicles and 43.1 ± 4.7, 135 ± 12, and 3400 ± 140 nM in the G_{i2}-reconstituted vesicles, respectively (mean ± standard error, four experiments). STA_2 binding to the receptor in both G_q- and G_{i2}-reconstituted vesicles was saturable at approximately 1 μM ligand. These binding affinities of S-145 and STA_2 were almost identical to those found for the receptor reconstituted alone in the vesicles (data not shown).

We next examined the effects of STA_2 on [^{35}S]GTPγS binding to the G proteins. The time course of [^{35}S]GTPγS binding to G_q reconstituted with the receptor in the vesicles is shown in Fig. 3A. The basal binding rate was very low, the rate was decreased further when vesicles were incubated with S-145, which indicates that G_q has low affinity for GTPγS without agonist stimulation, and the rate was decreased further by antagonist. The addition of STA_2 stimulated binding in a time-dependent fashion. The initial binding rate was increased 20–30-fold over the basal rate by 10 μM STA_2, and binding reached a plateau of 6.1 mol of [^{35}S]GTPγS/mol of receptor at 30 min,

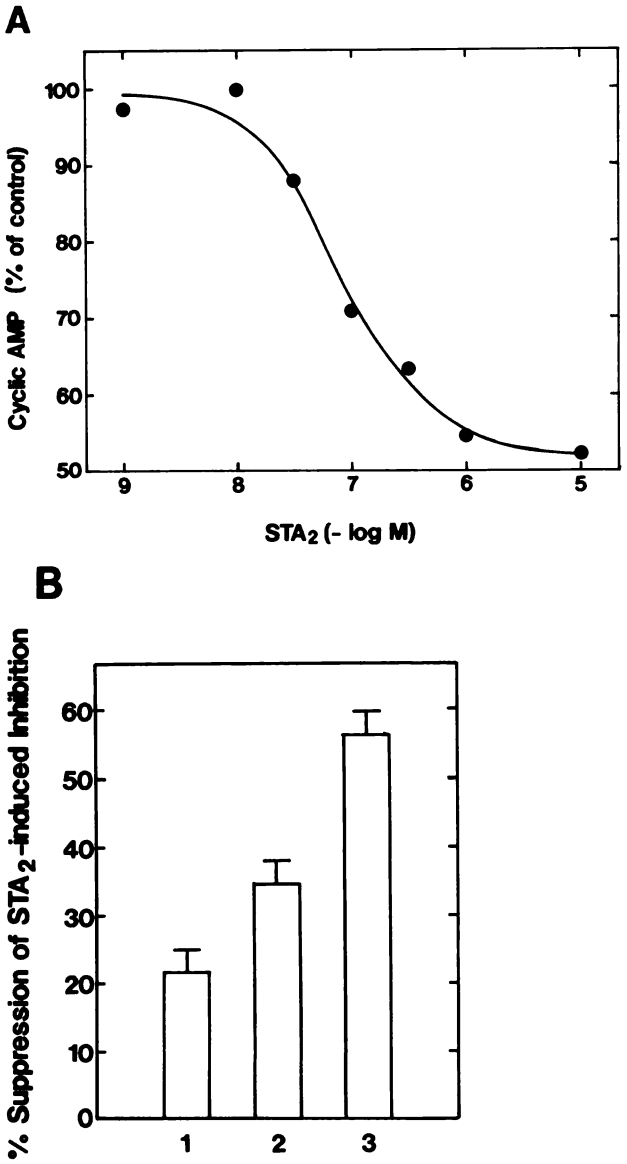

Fig. 7. Inhibition by STA_2 of the iloprost-induced platelet cAMP increase and reversal of the inhibition by S-145. A, Washed platelet suspensions were incubated with 3 nM iloprost and various concentrations of STA_2, and cAMP contents were measured as described in Experimental Procedures (mean values, three experiments). Iloprost (3 nM) alone raised cAMP levels from 8.6 to 242.6 pmol/10^8 platelets. B, Washed platelet suspensions were incubated with 3 nM iloprost, 0.1 μM STA_2, and either 0.1 μM (*column 1*), 1 μM (*column 2*), or 10 μM (*column 3*) S-145. cAMP contents were measured as described in Experimental Procedures (means ± standard errors, four experiments).

which shows that the agonist-liganded TXA_2 receptor acted catalytically. As shown in Fig. 3B, this stimulation of GTPγS binding to G_q was dependent on the STA_2 concentration. The binding, measured at 30 min of incubation, was half-maximal at 60 nM and reached a plateau at 1 μM STA_2, which correlated well with the binding of STA_2 to the receptor in the reconstituted system.

On the other hand, the rate of [^{35}S]GTPγS binding to G_{i2} in the reconstituted vesicles was only slightly increased by the agonist-liganded receptor (Fig. 4A). This was apparently due to the high basal rate of GTPγS binding to G_{i2}. We therefore added GDP to the assay mixture to block basal binding (28). After the addition of 1 μM GDP, STA_2 stimulated the rate of GTPγS binding to G_{i2} in a time- and concentration-dependent manner. The time course of GTPγS binding to reconstituted G_{i2} is shown in Fig. 4B. As was seen with the G_q-reconstituted vesicles, S-145 decreased the basal rate of binding. On the other hand, 10 μM STA_2 stimulated the initial rate of binding 1.5–2-fold over the basal rate. The binding did not reach a plateau, and 0.7 mol of GTPγS/mol of receptor was bound at 30 min. This apparently low turnover number and low rate of GTPγS binding in the G_{i2}-reconstituted vesicles may be due to agonist-stimulated GDP-GDP exchange in the presence of a high GDP concentration (about 100-fold higher than the [^{35}S]GTPγS concentration). The true turnover number would be much higher. The agonist-stimulated GTPγS binding (Fig. 4C), measured after 30 min of incubation, was half-maximal at 150 nM and reached a plateau at 1 μM STA_2. This concentration dependency is almost identical to that seen with the G_q-reconstituted vesicles. The results described above, taken together, show that the TXA_2 receptor functionally couples to both G_q and G_{i2} in the reconstitution system.

We next examined agonist-induced GTP hydrolysis in the reconstituted vesicles. When STA_2 was added, GTP hydrolysis increased linearly with time in both the G_q- and G_{i2}-reconstituted vesicles (Fig. 5). The rate of agonist-stimulated [^{32}P]P_i release was 2.21/min·receptor and 0.87/min·receptor with the G_q- and G_{i2}-reconstituted vesicles, respectively. These results clearly show that the agonist-liganded receptor catalytically stimulates the binding of [^{32}P]GTP to both G_q and G_{i2} in the reconstituted vesicles. We next measured the rate constants of hydrolysis of bound GTP in the presence of an agonist or an antagonist in the G_q- and G_{i2}-reconstituted vesicles (Fig. 6). After the vesicles were preincubated with 0.1 μM [γ-^{32}P]GTP for 3 min, 1 mM unlabeled GTP was added and the liberated [^{32}P]P_i was measured. The rate constants for hydrolysis of bound GTP (k_{cat}) were 0.87 ± 0.21 min^{-1} for G_q and 2.42 ± 0.12 min^{-1} for G_{i2} in the presence of the agonist STA_2 (mean ± standard error, three experiments). These values are consistent with those reported previously for $G_{q/11}$ (19) and G_i or G_o (28, 29). The k_{cat} value in the presence of S-145 was 2.37 ± 0.17 min^{-1} for G_{i2} (mean ± standard error, three experiments), which is almost identical to that measured in the presence of an agonist. The k_{cat} value in the presence of S-145, however, could not be determined for G_q because this protein bound very little GTP under these conditions.

STA_2-induced inhibition of platelet cAMP responses. The results described above showed that the TXA_2 receptor couples functionally to G_{i2} in the reconstituted system. To explore the physiological relevance of this *in vitro* finding, we examined the effect of STA_2 on platelet adenylate cyclase. As shown in Fig. 7A, STA_2 inhibited, in a concentration-dependent manner, the increase in platelet cAMP levels induced by 3 nM iloprost, a prostaglandin I_2 agonist. This inhibitory effect was half-maximal at about 90 nM STA_2 and maximal at about 1 μM, which correlated well with previously reported values of STA_2 binding to the TXA_2 receptor (24). The inhibitory effect of STA_2 was antagonized in a concentration-dependent manner by S-145, suggesting that it was a TXA_2 receptor-mediated process (Fig. 7B). These results suggest that the TXA_2 receptor couples to G_i, which then negatively regulates adenylate cyclase in platelets.

Discussion

The present study reports the first reconstitution of partially purified TXA_2 receptor and purified G proteins, G_q and G_{i2}. As shown in previous reports on m1 and m2 muscarinic receptors (18, 19), reconstitution analysis examines the selectivity of the coupling between receptors and G proteins, and the results correlate well with the physiological specificity of the two molecules. In this reconstitution system, a TXA_2 agonist, STA_2, stimulated both [^{35}S]GTPγS binding to and [γ-^{32}P]GTP hydrolysis by G_q and G_{i2}. The turnover numbers for GDP-GTP exchange, as calculated by the steady state rate for GTP hydrolysis, were 2.21 and 0.87/min·receptor for G_q and G_{i2}, respectively, under the present assay conditions with 0.1 μM GTP. This functional coupling of the TXA_2 receptor to two different species of G proteins, G_q and G_{i2}, may explain, at least in part, the multiplicity of signal transduction through the TXA_2 receptor. It is consistent with the previous findings that TXA_2 induces phosphatidylinositol turnover in a pertussis toxin-insensitive manner. It is also consistent with some reports that the TXA_2 receptor negatively regulates adenylate cyclase activity (30, 31), which was confirmed in the present study (Fig. 7).

Coupling of the TXA_2 receptor to G_{i2} has several implications. Some agonists of G protein-coupled receptors, such as thrombin (32), lysophosphatidic acid (33), and acetylcholine (34), are known to promote cell proliferation in a pertussis toxin-sensitive manner, and this signaling pathway leads to the activation of $p21^{ras}$, Raf, and mitogen-activated protein kinase (33, 35). Furthermore, *gip2* oncogene product, which is a GTPase-deficient G_{i2} α subunit mutant polypeptide, can transform Rat 1a fibroblasts, producing constitutive activation of mitogen-activated protein kinase (36). These results suggest the involvement of G_i in a signaling pathway by which G protein-coupled receptors stimulate cell proliferation. In some types of cells TXA_2 also promotes cell proliferation (14–16), apparently by stimulating mitogen-activated protein kinase (35). G_{i2} likely participates in signal transduction in these cells. It is also known that PLA_2 is activated by phosphorylation by mitogen-activated protein kinase (37). Fuse *et al.* (38) reported impaired PLC activation despite normal PLA_2 activation by TXA_2 in platelets from a patient with a mild bleeding disorder. They suggested that G proteins other than $G_{q/11}$ activate PLA_2, and G_{i2} is a possible candidate.

Our recent cloning studies showed that the human TXA_2 receptor gene exists as a single copy (39). On the other hand, there are pharmacological studies suggesting the presence of subtypes of the TXA_2 receptor (40). In light of the detection of a single TXA_2 receptor gene, these pharmacological findings may reflect different states of a single species of receptor coupling to different G proteins. Although the binding affinities of several analogues tested were not significantly different for receptors coupling to G_q and G_{i2}, it is likely that certain agonists activate the two G proteins with differential efficacy. It is also probable that the TXA_2 receptor can couple to G proteins other than G_q and G_{i2}. These possibilities will be examined in a future study.

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