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The G_{12} Family of G Proteins as a Reporter of Thromboxane A_2 Receptor Activity

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ABSTRACT

Despite advances in the understanding of pathways regulated by the G_{12} family of heterotrimeric G proteins, much regarding the engagement of this family by receptors remains unclear. We explore here, using the thromboxane A_2 receptor TPα, the ability of G_{12} and G_{13} to report differences in the potency and efficacy of receptor ligands. We were interested especially in the potential of the isoprostane 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), among other ligands examined, to activate G_{12} and G_{13} through TPα explicitly. We were also interested in the functionality of TPα-Gα fusion proteins germane to G_{12} and G_{13}. Using fusion proteins in *Spodoptera frugiperda* (Sf9) cells and independently expressed proteins in human embryonic kidney 293 cells, and using guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding to evaluate Gα activation directly, we found for Gα_{13} that no ligand tested, including 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) and a purported antagonist (pinane thromboxane A_2), was silent. The activity of agonists was especially pronounced when evaluated for TPα-Gα_{13} and in the context of receptor reserve. Agonist activity for 8-iso-$PGF_{2\alpha}$ was diminished and that for pinane thromboxane A_2 nonexistent when Gα_{12} was the reporter. These data establish that G_{12} and G_{13} can report differentially potency and efficacy and underscore the relevance of receptor and G protein context.

The G_{12} family of heterotrimeric G proteins, comprising G_{12} and G_{13} in vertebrates, has received considerable attention for its roles in cell contractility, motility, and proliferation (for review, see Riobo and Manning, 2005). Specific functions for this family have been deduced through the actions of constitutively active Gα subunits, effects of deleting one or both Gα genes, effects of dominant-negative molecules, and interactions of the Gα subunits with other proteins. Despite advances in understanding the pathways regulated by the G_{12} family, however, much regarding the engagement of this family by agonists remains unclear. At a basic level, the extent to which G_{12} and G_{13} can report differences among agonists, for example, in terms of potency and efficacy, is entirely unknown, nor can it be easily predicted given the unusual properties of the respective Gα subunits (Singer et al., 1994; Kozasa and Gilman, 1995). One of the problems in evaluating the dynamics of signaling through G_{12} and G_{13} is that enzymes or ion channels uniquely regulated by the two proteins and whose activities are easily measured have not yet been identified. A related problem is that receptors having the capacity to couple to G_{12} and/or G_{13} invariably couple to other G proteins as well. The analysis of proximal signaling so important to the modeling of receptor function in the context of other G proteins, therefore, has proven difficult for the G_{12} family and, except for measurements of frank activation, has not been approached.

Thromboxane A_2 is a member of the prostaglandin family of lipid mediators generated after cyclooxygenase-catalyzed conversion of arachidonic acid (Smith et al., 2000). As the principal product of the cyclooxygenase-1 isoform in platelets, thromboxane A_2 causes contraction and proliferation of vascular smooth muscle cells and activation of platelets, in the latter case evoking aggregation and amplifying the action of other platelet stimuli. These actions are concordant with studies implicating thromboxane A_2 in the pathology of a variety of cardiovascular diseases, including atherosclerosis (Kobayashi et al., 2004; Egan et al., 2005), stenosis after vascular injury (Cheng et al., 2002; Rudic et al., 2005), and hypertension (Francois et al., 2004). The biological actions of thromboxane A_2 are achieved through activation of one or both of its receptors, TPα and TPβ, which are produced

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ABBREVIATIONS: 8-iso-PGE_2, 8-iso-prostaglandin E_2; 8-iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$; SQ29548, [1*S*-[1α,2α(*Z*),3α,4α]]-7-[3-[[2-[(phenyl amino)carbonyl]hydrazine]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; U46619, 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5*Z*,13*E*-dien-1-oic acid; PTA_2, pinane-thromboxane A_2; U44069, 9,11-dideoxy-9α,11α-epoxymethano-prosta-5*Z*,13*E*-dien-1-oic acid; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; HA, the sequence YPYDVDPDYA from hemagglutinin; HEK, human embryonic kidney; TP, thromboxane A_2 receptor.

through differential splicing of a single gene product (Raychowdhury et al., 1994). Within the context of G protein signaling, TPα, the predominant isoform in most if not all tissues studied (Miggin and Kinsella, 1998), is the more extensively characterized. TPα exhibits the capacity in platelets to activate G_q, G_{12}, and G_{13} (Offermanns et al., 1994).

Issues of agonism at TPα and/or TPβ in general rest on effector activities or cell responses where G_q plays a prominent role. Of interest in this regard are the actions of isoprostanes, prostaglandin-like compounds produced primarily by oxygen free radical-induced peroxidation of arachidonic acid (Montuschi et al., 2004). Isoprostanes are in vivo markers of oxidant stress and are elevated coincident with perturbed cardiovascular function. The effects of two such isoprostanes, 8-iso-prostaglandin E_2 (8-iso-PGE_2) and 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), are blocked by TP receptor antagonists, suggesting a role for these compounds as TP receptor agonists (for review, see Janssen, 2001; Montuschi et al., 2004). Indeed, 8-iso-$PGF_{2\alpha}$ mobilizes Ca^{2+} in HEK293 cells when TPα is introduced (Kinsella et al., 1997). Controversy remains, however, as to the identity of the receptor(s) activated by these mediators. Neither 8-iso-PGE_2 nor 8-iso-$PGF_{2\alpha}$ consistently displaces the binding of the TP antagonist [^{3}H]SQ29548 efficiently (Pratico et al., 1996; Wilson et al., 2004), nor does 8-iso-$PGF_{2\alpha}$ mimic the actions of U46619, a thromboxane A_2 mimetic, in several cells (Morrow et al., 1992), including platelets (Pratico et al., 1996). Furthermore, in contrast to HEK293 cells (Kinsella et al., 1997), expression of TPα in Chinese hamster ovary cells does not reconstitute mobilization of Ca^{2+} by 8-iso-$PGF_{2\alpha}$ (Weber and Markillie, 2003). No study has examined the potential of isoprostanes, through TPα, to activate G_{12} and G_{13} or has directly evaluated TPα apart from the modifying influences of endogenously expressed receptors, for example, those in mammalian cells that might change functionality through receptor heterodimerization or competition for G proteins (Wilson et al., 2004).

We examine here, using TPα, the extent to which potency and efficacy for ligands that operate through G_{12} and G_{13} can be evaluated. We address specifically the question of 8-iso-$PGF_{2\alpha}$ signaling through TPα and the capacity of other ligands as well to signal as a function of the G protein examined and context of receptor expression.

Materials and Methods

Materials. U46619, SQ29548, pinane thromboxane A_2 (PTA_2), U44069, 8-iso-PGE_2, and 8-iso-$PGF_{2\alpha}$ were purchased from Cayman Chemical (Ann Arbor, MI). Protein A-Sepharose, aprotinin, normal rabbit serum, GTP, and GDP were purchased from Sigma-Aldrich (St. Louis, MO). Pansorbin cells and Nonidet P-40 were purchased from Fisher Scientific (Pittsburgh, PA). [^{35}S]GTPγS (1250 Ci/mmol) and [^{3}H]SQ29548 (58 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Sf900-II and Grace's insect medium (supplemented) were purchased from Invitrogen (Carlsbad, CA). cDNA for the hemagglutinin (HA)-tagged human TPα receptor DNA was described previously (Wilson et al., 2004); the tag is at the N terminus. cDNAs for human $G\alpha_{12}$ and $G\alpha_{13}$ were purchased from Guthrie (Sayre, PA). Rabbit antisera for $G\alpha_{12}$ and $G\alpha_{13}$ were produced using peptides corresponding to the C-terminal 10 residues of the two proteins (Butkerait et al., 1995; Windh et al., 1999). The HA-directed monoclonal antibody HA.11 was purchased from Covance (Berkeley, CA).

Construction of Receptor-Gα Fusion Proteins. TPα-$G\alpha_{13}$ was constructed by first introducing a BamH1 restriction site immediately 5′ to the start codon of the (HA-tagged) TPα cDNA and substituting the stop codon with an EcoR1 site by polymerase chain reactions. BamH1/Kpn and Kpn/EcoR1 fragments were subcloned into pcDNA(zeo) to reconstitute full-length TPα cDNA, absent the stop codon, bounded by the two restriction sites. An EcoR1 site was introduced immediately 5′ to codon 2 of the $G\alpha_{13}$ cDNA, eliminating the start codon. An EcoR1/XbaI restriction fragment, containing full-length $G\alpha_{13}$ absent the start codon, was subcloned into the pcDNA(zeo) containing HA-TPα to form TPα-$G\alpha_{13}$ cDNA. The TPα-$G\alpha_{13}$ cDNA was subcloned into pcDNA3.1 using BamH1/BamH1 and then XhoI/XbaI digests. It was also subcloned into pFastbac using BamH1/BamH1 and then NotI/XbaI digests from TPα-$G\alpha_{13}$ in pcDNA3.1. TPα-$G\alpha_{12}$ was formed by appending an EcoR1 site immediately 5′ to codon 2 of $G\alpha_{12}$ cDNA and then subcloning the EcoR1/XbaI fragment into pFastbac containing TPα-$G\alpha_{13}$, with elimination of the $G\alpha_{13}$ cDNA. The TPα-$G\alpha_{12}$ cDNA was formed in pcDNA3.1 by subcloning a NotI/XbaI fragment from pFastbac containing TPα-$G\alpha_{12}$ into pcDNA3.1 containing HA-TPα. Production of the recombinant baculoviruses was performed using the Bac-To-Bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions.

Cell Culture and Membrane Preparation. *Spodoptera frugiperda* (Sf9) cells (American Type Culture Collection, Manassas, VA) were maintained in suspension culture in Grace's insect medium containing 10% heat-inactivated fetal bovine serum and 0.1% Pluronic F-68 at 27°C. For infection with recombinant baculoviruses, the cells were subcultured in monolayer and infected at a multiplicity usually of 1. The medium was replaced 16 h after infection with Sf900-II optimized serum-free medium (Invitrogen). The cells were harvested at 48 h, washed three times with 0.9% NaCl, and resuspended in 1 ml of ice-cold 20 mM HEPES, pH 8.0, 1 mM EDTA, 0.11% aprotinin, 0.02% leupeptin, and 0.1% phenylmethylsufonyl fluoride. After 5 min on ice, cells were homogenized by repeated passage through a 26-gauge needle. Homogenates were centrifuged at 110*g* for 5 min, and the resultant supernatants were centrifuged at 20,800*g* for 30 min at 4°C. The final pellets (membrane) were resuspended at ~3 mg/ml protein.

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C with 5% CO_2. HEK293 cells stably expressing TPα (4.8 pmol of receptor/mg of membrane protein; Wilson et al., 2004) were maintained in the above-mentioned medium but containing 1 mg/ml Geneticin (G418). Membranes were made from HEK293 cells in essentially the same manner as for Sf9 cells.

[^{3}H]SQ29548 Binding. Membranes (20 μg of protein/assay point) were incubated with specified concentrations of [^{3}H]SQ29548 in 20 mM HEPES, pH 7.4, 2 mM EDTA, and 5 mM NaCl for 30 min at 30°C. The incubation volume was 0.1 ml. Reactions were terminated by dilution with 10 mM HEPES, pH 7.4, and 0.01% bovine serum albumin at 0°C and rapid filtration over Whatman GF/C filters presoaked in the same buffer. The filters were washed three times with the same buffer at 0°C and dried. Filter-bound radioactivity was determined by scintillation spectrometry. Nonspecific binding, generally less that 10% at K_d, was defined as the binding of radioligand in the presence of 250 μM SQ29548.

Assay of [^{35}S]GTPγS Binding. The assay for agonist-promoted binding of [^{35}S]GTPγS to $G\alpha_{12}$ and $G\alpha_{13}$ was performed essentially as described previously (Windh et al., 1999). Membranes (20 μg of protein/assay point) were resuspended in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 20 mM $MgCl_2$, 0.1 μM GDP, and 5 nM [^{35}S]GTPγS in 1.5-ml Eppendorf Microfuge tubes on ice. Ligands, if any, were added, and the tubes were transferred immediately to a 30°C water bath for times ranging from 1 to 40 min as noted. The incubation was terminated by adding 600 μl of ice-cold 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40,

0.33% aprotinin, 0.1 mM GDP, and 0.1 mM GTP. The extract was transferred to a microcentrifuge tube containing 2 μl of nonimmune serum preincubated with 100 μl of a 12% suspension of Pansorbin cells. Nonspecifically bound proteins were removed after 20 min by centrifugation. The extract was then incubated for 1 h at 4°C with 10 μl of a Gα-directed antiserum or HA-directed antibody, or nonimmune serum, all of which had been preincubated with 100 μl of a 5% suspension of protein A-Sepharose. Immunoprecipitates were collected and washed three times in the extraction buffer, then once in the buffer without detergent, and then boiled in 0.5 ml of 0.5% SDS followed by addition of 5.2 ml of Ecolite+ (MP Biomedicals, Irvine, CA). The samples were analyzed directly by scintillation spectrometry. Counts obtained with nonimmune serum, representing nonspecifically bound radiolabel and generally in the range of 50 to 200 cpm, were subtracted before any portrayal of the data.

Miscellaneous. Western blots were performed by SDS-polyacrylamide gel (12% acrylamide) electrophoresis followed by transfer of protein to Immobilon-P membranes (Millipore, Billerica, MA); block of nonspecific binding sites with 5% nonfat milk in 0.1 M Tris-HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween 20; incubation with primary antisera or antibodies, generally at 1:1000 dilutions; washing in the same buffer but without nonfat milk; incubation with horseradish peroxidase-conjugated secondary antibodies; washing; and visualization with enhanced chemiluminescence Western blotting detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). [^{3}H]SQ29548 and [^{35}S]GTPγS binding were evaluated by nonlinear regression using Prism (GraphPad Software Inc., San Diego, CA). Statistical differences were determined using Student's t test, with $p < 0.05$ signifying a difference, or using 95% confidence intervals, with nonoverlapping intervals signifying a difference.

Results

To investigate the properties of transduction through members of the G_{12} family of heterotrimeric G proteins, we chose to focus on the signaling effected through the thromboxane A_2 receptor TPα. TPα couples to both G_{12} and G_{13} and has been studied extensively in the context of platelet and vascular smooth muscle cell function. We examined first the activation of G_{13}, which provided more easily discerned signals for all the ligands tested (see below).

We explored the expression and functionality of a TPα-Gα_{13} fusion protein introduced into Sf9 cells. Receptor-Gα fusion proteins fix the ratio of receptor and Gα at 1:1 and have been well documented for receptors communicating with Gα_s and Gα_i (Siefert et al., 1999; Milligan, 2000). Sf9 cells contain few, if any, receptors that interfere with the analysis of those introduced by means of recombinant baculoviruses (Windh and Manning, 2002). Analysis by Western blotting of membranes from cells with an antibody directed toward the N-terminal HA epitope as well as with an antibody directed toward the C-terminal 10 residues of Gα_{13} confirmed expression of the protein after infection (Fig. 1). The fusion protein had an electrophoretic mobility corresponding to ~80 kDa, close to the predicted molecular mass of 82.7 kDa. Saturation binding with the radiolabeled antagonist [^{3}H]SQ29548 revealed a B_{max} of 2 to 4 pmol/mg of membrane protein and a K_d of 57 ± 11 nM (n = 3). The K_d was similar to that observed when the receptor was expressed without the appended Gα (33 ± 2 nM; n = 3; not shown) or when it was overexpressed in HEK293 cells (35 nM) (Wilson et al., 2004). No binding was observed in the membranes without introduction of the fusion protein.

[^{35}S]GTPγS-binding assays with Sf9 cell membranes containing TPα-Gα_{13} revealed that the Gα subunit can be activated by U46619, a prototypic TP agonist (Fig. 2). In these and other experiments, TPα-Gα_{13} was immunoprecipitated after incubation of membranes with [^{35}S]GTPγS ± agonist with a Gα_{13}-specific antiserum; immunoprecipitation is required to achieve adequate resolution of the Gα-specific signal from nonspecific background (Windh and Manning, 2002). As evident in Fig. 2 (top), the rate of binding of [^{35}S]GTPγS to Gα_{13} was relatively high, departing from linearity within 2 min and reaching a maximum within 5 min. Thus, it was necessary in experiments evaluating concentration-response relationships to operate at the earliest time point possible, 1 min. Figure 2 (middle) illustrates an experiment of this nature, wherein U46619 effected a 10-fold increase in [^{35}S]GTPγS binding with an EC_{50} of approximately 0.7 μM. Data for several experiments combined are shown in Fig. 2 (bottom) and Table 1. The assay was carried out successfully as well by using an antibody directed toward the N-terminal HA tag of the fusion protein for immunoprecipitation (data not shown). The ability of a fusion protein com-

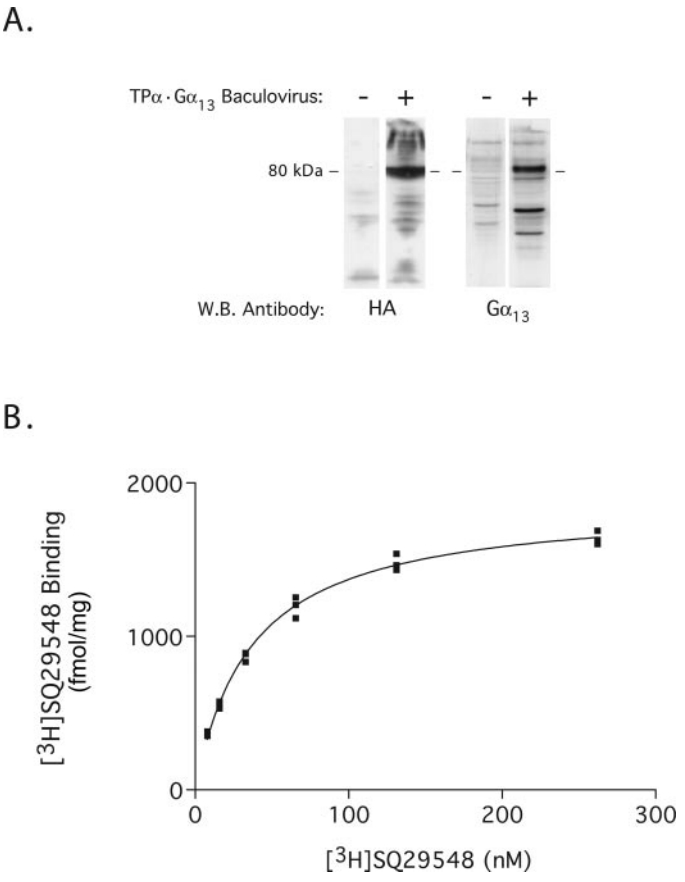

Fig. 1. Expression of TPα-Gα_{13} in Sf9 cells. A, membranes were prepared from Sf9 cells that were, or were not, infected with a recombinant baculovirus encoding TPα-Gα_{13}. The membranes (30 μg of protein) were analyzed by Western blotting (W.B.) with a monoclonal antibody against the HA epitope N terminal to the receptor or with a rabbit antiserum generated against the C-terminal 10 residues of Gα_{13}. The fusion protein has an apparent molecular mass of approximately 80 kDa, calculated from its migration relative to standards. The more rapidly migrating bands observed with the Gα antiserum were evident after infection with irrelevant baculoviruses as well. B, membranes (20 μg of protein) from Sf9 cells expressing TPα-Gα_{13} were incubated in triplicate for 30 min at 30°C with the indicated concentrations of [^{3}H]SQ29548. Bound radiolabel was determined after filtration. Nonspecific binding (less than 10% at K_d) was determined with 250 μM SQ29548 and was subtracted from total binding. Each of the experiments was representative of several others.

prising receptor and Gα subunit to signal can therefore be extended to the G_{12} family, and G_{13} in particular, based on a clearly defined, graded response of TPα-$G\alpha_{13}$ to U46619.

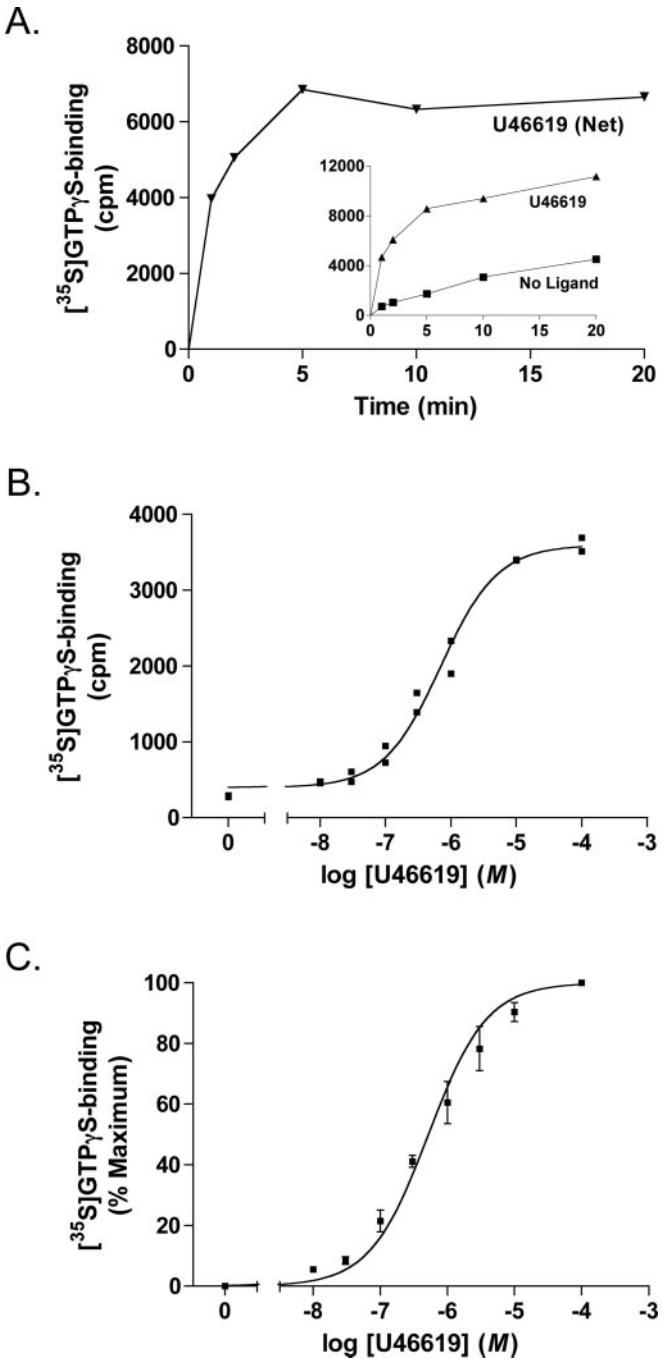

Fig. 2. Activation of $G\alpha_{13}$ by U46619. A, membranes from Sf9 cells expressing TPα-$G\alpha_{13}$ were incubated for 1 to 20 min at 30°C with [^{35}S]GTPγS with or without 100 μM U46619. The membranes were solubilized, immunoprecipitation was carried out with an antiserum directed toward the C terminus of $G\alpha_{13}$, and bound radioactivity was evaluated by scintillation spectrometry. The graph portrays ligand-dependent binding (i.e., the binding obtained with U46619 minus that obtained without U46619) [U46619 (Net)]. B, an individual experiment, in which membranes from Sf9 cells expressing TPα-$G\alpha_{13}$ were incubated in duplicate for 1 min at 30°C with [^{35}S]GTPγS and the indicated concentrations of U46619. The membranes were processed as in A for determination of bound radioactivity. C, U46619-effected binding was evaluated as in B and normalized by setting agonist-independent binding to 0% and maximum U46619-effected binding to 100% (n = 3 independent experiments).

A question of interest, in part bearing on the functionality of the fusion protein, is the extent to which $G\alpha_{13}$ can report differences in potencies and efficacies among ligands that operate through TPα, because the properties of such ligands are commonly referenced to events, carried out through TPα or not, where G_q instead plays a prominent role. We evaluated five compounds in addition to U46619: U44069, considered like U46619, a full agonist; the isoprostanes 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$, whose efficacy, if operating through TPα at all, is uncertain; and PTA_2 and SQ29548, reportedly antagonists. Using again TPα-$G\alpha_{13}$ in Sf9 cells and [^{35}S]GTPγS binding, we found that all ligands but SQ29548 promoted activation of $G\alpha_{13}$ (Fig. 3). Differences in maximal binding were evident. No agonist exhibited a maximal activity (E_{max}) equal to that of U46619 (Table 1). Those of U44069 and 8-iso-PGE_2 were approximately 80%, and those of 8-iso-$PGF_{2\alpha}$ and PTA_2 were approximately 60%, the E_{max} of U46619. The rank order of potencies was U44069 > U46619 ~ PTA_2 > 8-iso-PGE_2 > 8-iso-$PGF_{2\alpha}$. Thus, $G\alpha_{13}$ can register differences in both efficacy and potency. The data also attest, for the first time, to the properties of isoprostanes as agonists for TPα as visualized through activation of $G\alpha_{13}$ and point, unexpectedly, as well to the property of PTA_2 as an agonist.

In contrast to the other ligands, SQ29548 did not exhibit the activity of an agonist (Fig. 3, bottom). Indeed, SQ29548 suppressed the small, but consistently obtained, degree of ligand-independent $G\alpha_{13}$ activation. Whether this effect represented neutral antagonism in the face of an endogenous agonist or inverse agonism could not be established. However, inhibition of the synthesis of cyclooxygenase-derived metabolites with aspirin did not modify activity (data not

TABLE 1

Potency and efficacy of ligands in promoting [^{35}S]GTPγS binding to TPα·$G\alpha_{12}$ and TPα·$G\alpha_{13}$

EC_{50} values were calculated by nonlinear regression performed on concentration-response data for TPα·Gα fusion proteins expressed in Sf9 cells and are provided here as means and 95% confidence intervals (CI). The maximal effect of a ligand on [^{35}S]GTPγS-binding (E_{max}) was also determined by nonlinear regression and is expressed relative to binding promoted by 100 μM U46619 ± 1 S.E. (n = 3); values for U44069, isoprostanes, and PTA_2 differ statistically ($P < 0.05$) from 1.0. Concentration-response experiments used assay times of 1 min ($G\alpha_{13}$) and 5 or 10 min ($G\alpha_{12}$), except for TP expressed endogenously in HEK293 cells, for which the assay time was 10 min.

	EC_{50}		E_{max} (Relative)
	Mean	95% CI	
	nM		
Sf9 cells			
TPα-$G\alpha_{13}$			
U46619	520	420–640	(1.0)
U44069	285	215–375	0.83 ± 0.02
8-iso-PGE_2	7200	4670–11,000	0.76 ± 0.03
8-iso-$PGF_{2\alpha}$	44,000	32,000–60,000	0.62 ± 0.02
PTA_2	700	495–995	0.59 ± 0.02
TPα-$G\alpha_{12}$			
U46619	870	680–1120	(1.0)
8-iso-$PGF_{2\alpha}$	—[a]	—	—
PTA_2	—	—	—
HEK293 cells			
Overexpressed TPα/endogenous $G\alpha_{13}$			
U46619	78	59–104	(1.0)
Endogenous TP/endogenous $G\alpha_{13}$			
U46619	295	160–535	(1.0)

[a] Lack of convergence (PTA_2) or a large confidence interval indicating lack of significance (8-iso-$PGF_{2\alpha}$).

shown), ruling out involvement of endogenous thromboxane A_2. The data for SQ29548 demonstrate that ligand-independent activity was not entirely because of guanine nucleotide exchange intrinsic to $G\alpha_{13}$, but rather to some form of receptor-effected activation of the $G\alpha$ subunit.

Given the activity observed with PTA_2, purportedly an antagonist, and with 8-iso-$PGF_{2\alpha}$, whose interaction with $TP\alpha$ has been subject to debate—and also given the novelty of the receptor-$G\alpha_{13}$ fusion protein itself—we felt obliged to evaluate a different mode of receptor and $G\alpha_{13}$ expression altogether. We turned to HEK293 cells in which $TP\alpha$ was (over)expressed stably. Here, the binding of [^{35}S]GTPγS was measured for $G\alpha_{13}$ endogenous to the cells. We found that the rate of binding effected by U46619 through overexpressed $TP\alpha$ was approximately the same as that noted for $TP\alpha$-$G\alpha_{13}$ (data not shown). The -fold increase in binding was also high (approximately 10-fold; Fig. 4A); however, the EC_{50}, 80 nM (Fig. 4B; Table 1), was less. As before, 8-iso-$PGF_{2\alpha}$ and PTA_2 exhibited significant activity (Fig. 4C). In fact, the activity of 8-iso-$PGF_{2\alpha}$ approached that of U46619.

The lower EC_{50} for the activation of $G\alpha_{13}$ through $TP\alpha$ overexpressed in HEK293 cells relative to that within $TP\alpha$-$G\alpha_{13}$ in Sf9 cells (80 versus 520 nM) implied spare receptors in the former case or a less than optimal coupling in the latter case. A small complement of TP endogenous to HEK293 cells has been reported previously (Kinsella et al., 1997). We therefore examined the activation of $G\alpha_{13}$ endogenous to these cells without superimposed expression of receptor (Fig. 4, D and E). The rate of binding was not evaluated in this instance; instead, a 10-min time point alone was used; the EC_{50} may therefore represent an underestimate. Regardless, the EC_{50} for activation of $G\alpha_{13}$ through endogenous TP under these conditions was 290 nM. Notwithstanding the precise identity of endogenous TP, the higher EC_{50} suggested that the overexpression of $TP\alpha$ in HEK293 cells created a context of spare receptors. We noted that 8-iso-$PGF_{2\alpha}$ retained agonist activity through endogenous TP (Fig. 4F); the actions of PTA_2 were more difficult to ascertain (see legend). We also noted that agonist-independent activity for HEK293 cell membranes without overexpressed $TP\alpha$ was higher than that for membranes with overexpressed $TP\alpha$ even when the same assay times (data not shown) were used. Western blots revealed that levels of $G\alpha_{13}$ were severalfold lower in the latter membranes, indicating that overexpression of the receptor might cause a compensatory adaptation, increasing the ratio of receptor to G protein still further.

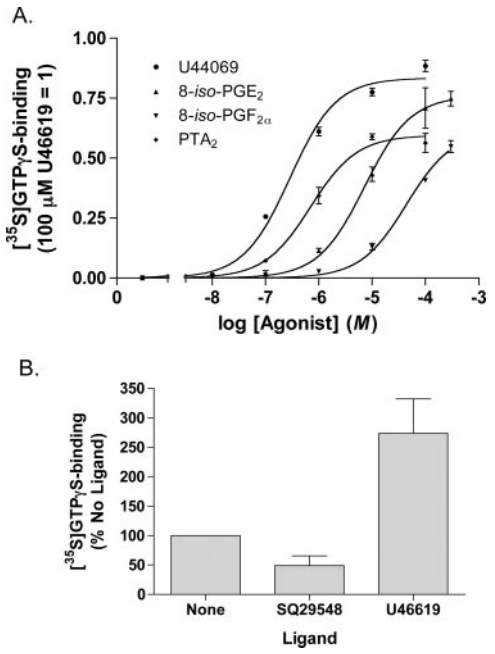

Fig. 3. Actions of several ligands on $G\alpha_{13}$ activity through $TP\alpha$. A, membranes from Sf9 cells expressing $TP\alpha$-$G\alpha_{13}$ were incubated for 1 min at 30°C with [^{35}S]GTPγS and the indicated concentrations of U44069, 8-iso-PGE_2, 8-iso-$PGF_{2\alpha}$, or PTA_2. The membranes were solubilized, and immunoprecipitation was carried out with the $G\alpha_{13}$-directed antiserum for quantitation of bound radiolabel. Binding was referenced to that obtained in the same experiments with 100 μM U46619, which was assigned an activity of 1.0, after subtraction of ligand-independent activity. B, membranes were incubated with 10 to 100 μM SQ29548 or 10 μM U46619 for 10 min. Activity is expressed as a percentage of ligand-independent [^{35}S]GTPγS binding; the percentage of increase with U46619 is lower than in other experiments because of the time point chosen. The changes from ligand-independent activity with SQ29548 and U46619 are significant ($p < 0.01$). $n = 3$; bars represent 1 S.E.M.

Data for purified G_{12} and G_{13} suggest equivalent [^{35}S]GTPγS-binding properties (Kozasa and Gilman, 1995); however, evidence for selectivity in activation by receptors is emerging (Riobo and Manning, 2005). We therefore wished to evaluate the activation of $G\alpha_{12}$ by $TP\alpha$. We returned to the fusion protein as a reporter, using now $TP\alpha$-$G\alpha_{12}$. Our intent was to compare the properties of $G\alpha_{12}$ to those of $G\alpha_{13}$ (i.e., $TP\alpha$-$G\alpha_{13}$) under equivalent conditions; $G\alpha_{12}$ is not expressed in HEK293 cells at detectable levels, nor would we have been able to factor the influence of slight differences in receptor/$G\alpha$ ratios or the influence of other receptors in these cells. Expression of $TP\alpha$-$G\alpha_{12}$ in Sf9 cells was comparable with that of $TP\alpha$-$G\alpha_{13}$, as was recognition of the two subunits by their respective antisera in Western blots (Fig. 5A; not shown). U46619 effected [^{35}S]GTPγS binding to $TP\alpha$-$G\alpha_{12}$, as it did to $TP\alpha$-$G\alpha_{13}$ (Fig. 5B). Of interest, the rate of binding for $TP\alpha$-$G\alpha_{12}$ was quite low, at most 5% of the maximum within 1 min. $G\alpha_{12}$ was activated, therefore, approximately 10-fold more slowly in response to U46619 than was $G\alpha_{13}$. The EC_{50} for activation was slightly greater than that for activation of $TP\alpha$-$G\alpha_{13}$ (870 versus 520 nM; Fig. 5C; Table 1). Thus, although the response of $G\alpha_{12}$ and $G\alpha_{13}$ to U46619-activated $TP\alpha$ differs in terms of the kinetics of guanine nucleotide binding, the sensitivity of the two subunits toward the receptor is similar. $TP\alpha$-$G\alpha_{12}$ was less sensitive than $TP\alpha$-$G\alpha_{13}$ to 8-iso-$PGF_{2\alpha}$; activation did not approach saturation, so an EC_{50} could not be calculated. It is remarkable that $TP\alpha$-$G\alpha_{12}$ was completely refractory to PTA_2. This observation suggested that $TP\alpha$-$G\alpha_{12}$, unlike $TP\alpha$-$G\alpha_{13}$, did not bind PTA_2 or could not respond to PTA_2 once the ligand was bound. PTA_2 antagonized the activation of $TP\alpha$-$G\alpha_{12}$ by U46619 (data not shown), indicating the latter possibility. The expression of activity on the part of 8-iso-$PGF_{2\alpha}$ and PTA_2 depends, therefore, on the nature of the $G\alpha$ used as the reporter.

Discussion

We have examined, using $TP\alpha$, the ability of $G\alpha_{12}$ and $G\alpha_{13}$ to report differences in potency and efficacy of ligands as monitored directly by [^{35}S]GTPγS binding. No previous study has evaluated parameters of agonist action as reported by the G_{12} family, which differs considerably in several bio-

chemical respects from other G protein families and whose activity is difficult to deduce without direct measurement. Nor has any previous study evaluated the possible utility of receptor-Gα fusion proteins as they pertain to this family. We find 1) the fusion proteins TPα-Gα_{12} and TPα-Gα_{13} are functional; 2) Gα_{12} and Gα_{13} report differences in potency and efficacy; 3) differences in the latter properties depend on which Gα subunit is evaluated and the circumstances of receptor and Gα expression; 4) 8-iso-$PGF_{2\alpha}$ is an agonist for TPα, but the unequivocal designation of it as such requires Gα_{13} as the reporter; 5) the properties of the "antagonist" PTA_2 in fact depend on the identity of the G protein; and 6) no ligand tested for TPα is silent, at least with Gα_{13} as the reporter.

The idea of evaluating R-Gα fusion proteins for Gα_{12} and Gα_{13} was based on successes achieved elsewhere for Gα_s and Gα_i (for review, see Siefert et al., 1999; Milligan, 2000). The invariant ratio of receptor to Gα subunit was attractive, because it theoretically obviates differences in expression that can complicate, ultimately, comparisons among different receptors and G proteins. Fusion proteins were used previously for Gα_{12} and Gα_{13} to help characterize the nature of Rac inhibition through the sphingosine 1-phosphate $S1P_2$ receptor (Sugimoto et al., 2003); however, activation of the Gα subunits was not measured directly and collateral activation of endogenous Gα_{12} and Gα_{13} was not precluded.

Ours is the first study to explicitly evaluate parameters of agonist action as translated by G_{12} or G_{13}. The EC_{50} for activation by U46619 of Gα_{12} and Gα_{13} fused to TPα was approximately 0.5 μM, consistent with EC_{50} values reported for a large number of U46619-effected phenomena and also consistent with the activation of Gα_{13} through TP endogenous to HEK293 cells (0.3 μM). The EC_{50} is higher than that reported for changes in platelet shape ($EC_{50} \sim 15$ nM; Ohkubo et al., 1996), a response unquestionably linked to G_{13} and not in some way achieved through G_q or a combination of G_q and G_{13} (Moers et al., 2003). The difference is conceivably due to the existence of spare receptors in platelets with respect to actin polymerization. Overexpression of TPα results in a greater potency of U46619 toward Gα_{13} activation itself, and it is not too speculative to assume limiting steps exist downstream of the G protein as well. The rank ordering of potencies for ligands as monitored through activation of at least Gα_{13} fused to TPα in Sf9 cells was in general consistent

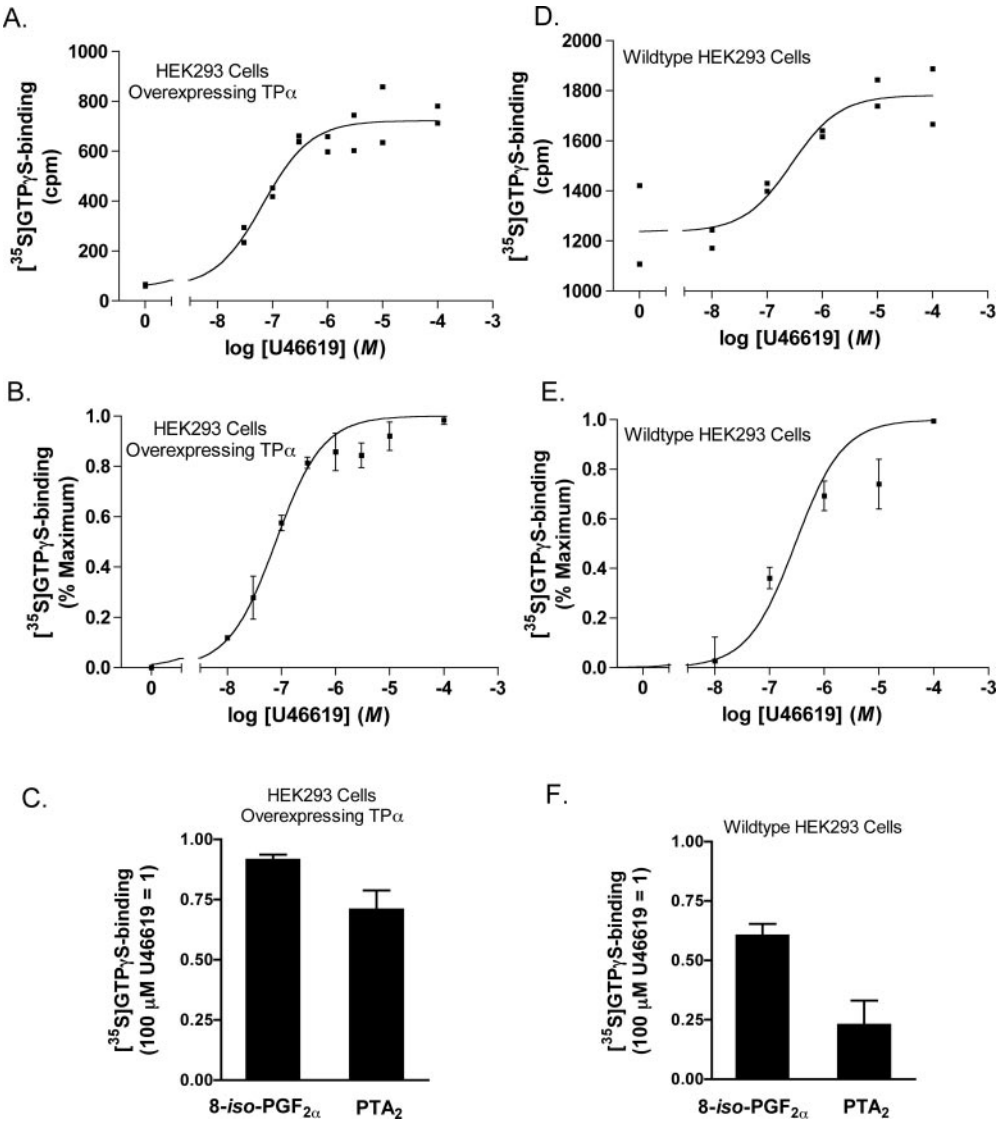

Fig. 4. Activation of Gα_{13} in HEK293 cells. Membranes from HEK293 cells (A–C) stably expressing heterologous TPα (approximately 5 pmol of receptor/mg of membrane protein) or wild-type HEK293 cells (D–F) were incubated with [^{35}S]GTPγS and U46619 at the indicated concentrations, 300 μM 8-iso-$PGF_{2\alpha}$, or 100 μM PTA_2. Assay times were 1 (A–C) or 10 (D–F) min. The membranes were solubilized, and immunoprecipitation of endogenous Gα_{13} was carried out with the Gα_{13}-directed antiserum followed by quantitation of bound radiolabel. A and D represent single experiments in duplicate, and B and E represent combined data from three to five experiments expressed as a fraction of binding achieved with 100 μM U46619. C and F represent binding promoted by 8-iso-$PGF_{2\alpha}$ and PTA_2 as a fraction of that promoted by 100 μM U46619 in paired experiments. In F, the binding promoted by PTA_2 is not statistically significant when evaluated for the stated concentration alone ($p < 0.05$ for a two-tailed t test), but it is statistically significant when concentration-response data are evaluated by nonlinear regression, wherein the 95% confidence interval for E_{max} is above 0.

with that of K_i values for displacement of [^{3}H]SQ29548 in several types of cells (Fukunaga et al., 1993a,b; Dorn et al., 1997; Cao et al., 2004; Wilson et al., 2004). There is no evidence, therefore, of selectivity among ligands in $G\alpha_{13}$ activation apart from what can be inferred through competitive binding.

Differences in maximal effects on $G\alpha_{13}$ among some of ligands were evident. In TPα-$G\alpha_{13}$, the ligands 8-iso-$PGF_{2\alpha}$ and PTA_2 exhibited approximately half the activity of U46619, whereas U44069 and 8-iso-PGE_2 exhibited approximately 80% the activity. Insofar as examined, relative differences were maintained in HEK293 cells, both those overexpressing TPα and those for which agonists worked through endogenous TP. $G\alpha_{13}$ can therefore resolve ligands according to efficacy. The higher activity of partial agonists in the case of overexpressed TPα relative to the same agonists using endogenous TP in HEK293 cells is likely due to spare receptors in the former instance. In contrast to the other ligands, SQ29548 did not activate $G\alpha_{13}$ but instead suppressed the small amount of ligand-independent activity evident for the fusion protein. A definitive demonstration of inverse agonism will require identifying a neutral antagonist; however, the possibility of inverse agonism for SQ29548 raises the spectre of receptor selection in a widely used radioligand binding assay, wherein [^{3}H]SQ29548 would prefer the noncoupled form of receptor.

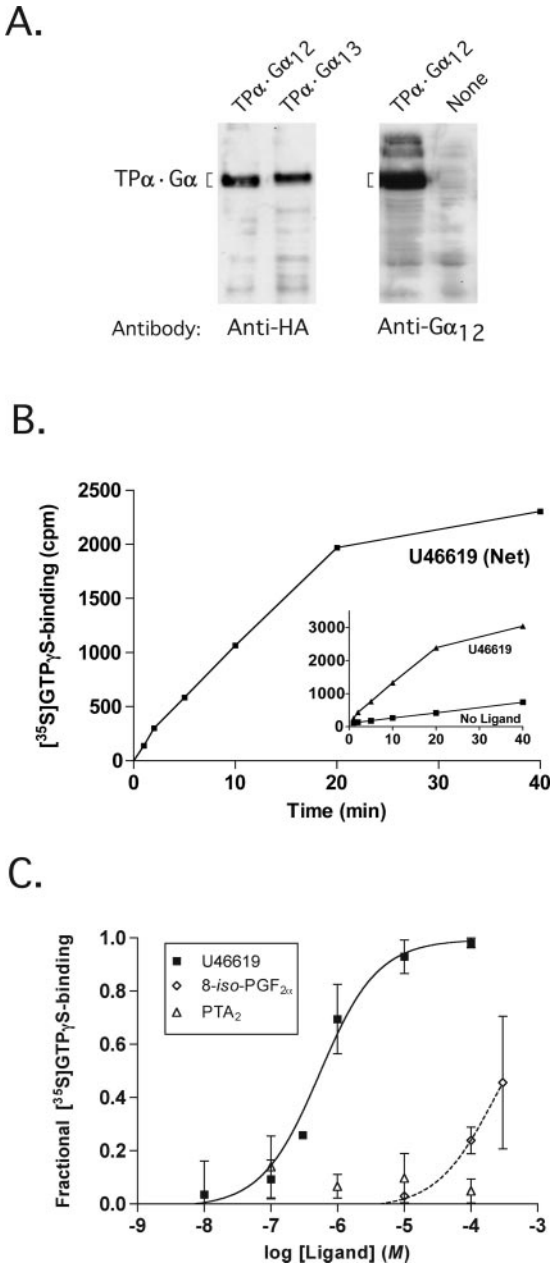

Fig. 5. Activation of $G\alpha_{12}$ through TPα. Membranes were prepared from Sf9 cells infected with recombinant baculovirus encoding TPα-$G\alpha_{12}$ (and, in A, TPα-$G\alpha_{13}$ also), or not infected, as noted. A, membranes (30 μg of protein/lane) were analyzed by Western blotting with a monoclonal antibody against the HA epitope or an antiserum against the C-terminal 10 residues of $G\alpha_{12}$. B, U46619-promoted binding of [^{35}S]GTPγS to TPα-$G\alpha_{12}$ as a function of time [U46619 (Net)], determined as binding obtained with 100 μM U46619 minus that obtained without U46619 (inset), using the $G\alpha_{12}$-directed antiserum for immunoprecipitation here and in C. C, U46619-, 8-iso-$PGF_{2\alpha}$-, and PTA_2-effected binding of [^{35}S]GTPγS to TPα-$G\alpha_{12}$, normalized by setting agonist-independent binding to 0 and maximum U46619-effected binding to 1 (n = 3–4; 10-min assay).

Obtaining an EC_{50} value that reflects best a ligand's actions through TPα requires recognition of how quickly $G\alpha_{13}$ can be activated. At high concentrations of U46619 (10–100 μM), the binding of [^{35}S]GTPγS for TPα-$G\alpha_{13}$ and for $G\alpha_{13}$ activated through overexpressed TPα in HEK293 cells departed from linearity within 2 min. The use of longer times in our assay, such as those used previously (Windh and Manning, 2002), resulted in a substantial decrease in EC_{50} because of lower concentrations of agonist having time to achieve saturation. This was not the case with TPα-$G\alpha_{12}$. [^{35}S]GTPγS binding for $G\alpha_{12}$ was far slower, and assay times up to 20 min provided satisfactory results in the evaluation of EC_{50} values. The nature of the difference in binding rates is unknown. Previous data with purified G_{12} and G_{13} provide no indication of differences in intrinsic GDP/[^{35}S]GTPγS exchange activity (Kozasa and Gilman, 1995). We think the difference in rates is likely to be a property of the G protein response to TPα. This difference, in the intact cell, may imply that G_{13} engages downstream pathways more rapidly than G_{12} in response to activation of the receptor. The difference may also imply a heightened sensitivity of G_{13} toward low concentrations of agonists or partial agonists, if the rate of activation is sufficiently high relative to the rate of deactivation (GTP hydrolysis) to cause a summation of activation events.

Differences in the kinetics of [^{35}S]GTPγS binding notwithstanding, $G\alpha_{12}$ and $G\alpha_{13}$ were similarly sensitive to activation by U46619 through TPα, as manifested in similar EC_{50} values. Of interest, the two G protein subunits perceived PTA_2 quite differently. PTA_2 is a potent agonist when viewed through $G\alpha_{13}$ but has no activity whatsoever when viewed through $G\alpha_{12}$. Our data suggest that the PTA_2-bound conformation of receptor is not recognized by $G\alpha_{12}$. The data, therefore, identify the potential for differential receptivity of Gα subunits toward ligands.

Isoprostanes arise through cyclooxygenase-independent, free radical-induced peroxidation of membrane lipids, and the F_2 isoprostanes in particular are likely to be pathological mediators of oxidative injury. Whether the isoprostanes are viewed to work through TPα has been complicated by inconsistent blockade with TP antagonists, inconsistent mimicry of U46619, and the possible existence of other receptors that mediate their actions. Our data emphasize the importance of context in sorting these possibilities out. 8-iso-$PGF_{2\alpha}$ un-

equivocally signals through G_{13}. The EC_{50} for 8-iso-$PGF_{2\alpha}$ (approximately 45 μM through TPα-Gα_{13}), however, is quite high. We suspect that, in vivo, the actions of 8-iso-$PGF_{2\alpha}$ through TPα would require significant receptor reserve or the aforementioned accumulation of activated G_{13} as a result of activation outpacing deactivation. It is interesting to note that dynamic changes in TP levels, perhaps influencing responsiveness to 8-iso-$PGF_{2\alpha}$, have been argued to occur in pathophysiological states, including oxidative stress (Valentin et al., 2004). Signaling for 8-iso-$PGF_{2\alpha}$ through the G_{12} family of G proteins may not be evident in cells where G_{12} predominates as a transducer. The sensitivity of a cell to 8-iso-$PGF_{2\alpha}$ will also depend on how well it activates, through TP receptors, G_q and whether 8-iso-$PGF_{2\alpha}$ might use other receptors more or less effectively as well.

Receptors that couple to G_{12} and G_{13} invariably couple to other G proteins additionally (Riobo and Manning, 2005) and would therefore seem to be especially relevant prospects for agonist-directed trafficking. The interplay between the G_q and G_{12} families, for example, at the level of protein kinase D (Yuan et al., 2001) and serum response factor activation (Sagi et al., 2001), has received considerable attention. The capacity to monitor potency and efficacy as reported by G_{12} and G_{13}, combined with the extension of these methods to other G proteins, provides an important step in the pursuit of trafficking.

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