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Prostaglandin $F_{2\alpha}$ Receptor-Dependent Regulation of Prostaglandin Transport

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ABSTRACT

Prostaglandin (PG) $F_{2\alpha}$ may act on its G protein-coupled receptor (FP) or be imported intracellularly via a transporter, which has high affinity for $PGF_{2\alpha}$ and PGE_2, but not prostacyclin (PGI_2). In cells overexpressing the epitope-tagged FP together with the human prostaglandin transporter (hPGT), stimulation of the FP with $PGF_{2\alpha}$ (1 nM-1 μM), or the less potent FP agonist, the isoprostane 8,12-*iso*-$iPF_{2\alpha}$-III, inhibited prostaglandin uptake via the hPGT. This effect was abolished by pretreatment of the cells with cholera toxin, but not with pertussis toxin. Furthermore, two dominant negative constructs directed against $G\alpha_s$ partially blocked FP-mediated regulation of hPGT function, also suggesting $G\alpha_s$ involvement in this phenomenon. Surprisingly, neither an activator (dibutyryl cyclic AMP) nor an inhibitor (H89) of cyclic AMP-dependent protein kinase had any effect on FP-mediated inhibition of hPGT activity. Furthermore, although $PGF_{2\alpha}$ increases intracellular cyclic AMP via $G\alpha_s$ activation, it does not induce phosphorylation of the transporter, excluding a role of cyclic AMP-dependent protein kinase in hPGT regulation. Activation of the PGI_2 receptor, which is also coupled to $G\alpha_s$, does not regulate hPGT activity, despite markedly augmenting adenylate cyclase activation. In conclusion, activation of the FP reduces intracellular import of prostaglandins for metabolic inactivation, increasing prostanoid availability for membrane receptor activation. This effect seems to be mediated via $G\alpha_s$, independent of adenylate cyclase and cyclic AMP-dependent protein kinase activation.

Prostaglandins (PGs) are evanescent biological mediators, which exert their effects by binding to specific receptors on cells in the immediate vicinity of their production. $PGF_{2\alpha}$ has diverse physiological actions in vitro. For instance, it causes vascular smooth muscle contraction (Csepli and Csapo, 1975), hypertrophy of cardiac myocytes (Mentz et al., 1988; Karmazyn, 1989; Kunapuli et al., 1998), and is critical to the induction of labor and parturition in vivo (Sugimoto et al., 1997). The actions of $PGF_{2\alpha}$ are mediated via a membrane receptor, the FP, which belongs to the G protein-coupled receptor (GPCR) superfamily. The FP activates phospholipase C in a pertussis toxin-insensitive manner (Gusovsky, 1991; Nakao et al., 1993; Quarles et al., 1993), suggesting interaction with member(s) of the Gq family of GTP-binding proteins (G proteins). Membrane receptors for PGs may also be activated by isoprostanes (Audoly et al., 2000), free radical-catalyzed PG isomers (Lawson et al., 1999). For example, the FP is activated in a specific and saturable manner by the isoprostane 8,12-*iso*-$iPF_{2\alpha}$-III (Kunapuli et al., 1998), previously known as 12-*iso*-$PGF_{2\alpha}$ (Rokach et al., 1997).

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Despite the absence of enzymatic activity in plasma capable of oxidizing $PGF_{2\alpha}$ to inactive metabolites, $PGF_{2\alpha}$ does not activate the FP on cells distant from its site of generation. Like PGE_2 and PGD_2, $PGF_{2\alpha}$ is cleared in a single passage through any of several vascular beds, such as the lung (Schuster, 1998). Although a carrier-mediated prostaglandin transport has long been suggested (Ferreira and Vane, 1967; McGiff et al., 1969; Piper et al., 1970), only one prostaglandin transporter (rat PGT) has been cloned from a rat library (Kanai et al., 1995). Subsequently, the human analog (hPGT) has been cloned from a kidney cDNA library (Lu et al., 1996) and its gene has been characterized (Lu and Schuster, 1998). The hPGT is a 643 amino acid protein, with 12 putative membrane-spanning domains (Lu et al., 1996). It is widely expressed (Lu et al., 1996) and is induced by laminar shear stress in endothelial cells (Topper et al., 1998). Both the hPGT and the rat PGT take up $PGF_{2\alpha}$, PGE_2, PGE_1, and PGD_2 with high affinity (Itoh et al., 1996; Lu et al., 1996), whereas they do not transport the prostacyclin analog iloprost (Lu et al., 1996). The stable hydrolysis product of thromboxane A_2, thromboxane B_2, is transported with low affinity (Itoh et al., 1996; Lu et al., 1996). Although it has

ABBREVIATIONS: PG, prostaglandin; FP, $PGF_{2\alpha}$ receptor; GPCR, G protein-coupled receptor; hPGT, human prostaglandin transporter; H89, *N*-[2-([*p*-bromocinnamyl]amino)ethyl-5-isoquinoline sulfonamide; dBcAMP, dibutyryl cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine; DIDS, 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid, disodium salt hydrate; HA, hemagglutinin; IP, PGI_2 receptor; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PGI_2, prostacyclin.

been suggested that the hPGT imports PGs for intracellular termination of their effects, it may also be involved in the export of newly synthesized PGs to act on membrane GPCRs (Chan et al., 1998).

Given that extracellular $PGF_{2\alpha}$ may ligate the FP or be imported via PGT, we investigated whether FP activation regulates hPGT activity. We report that FP activation by its cognate ligand $PGF_{2\alpha}$ and, to a lesser extent, by the isoprostane 8,12-*iso*-$iPF_{2\alpha}$-III, inhibits intracellular prostanoid import by the hPGT. This regulation seems to be mediated via the heterotrimeric G protein $G\alpha_s$, but independent of an increase in cyclic AMP levels or cyclic AMP-dependent protein kinase activation.

Experimental Procedures

Materials. All the cell culture media and G418 were purchased from Life Technologies Inc. (Gaithersburg, MD). Zeocin was purchased from Invitrogen (Carlsbad, CA). The anion exchange resin AG 1-X8 (formate form, 200–400 mesh) and 30% acrylamide/bisacrylamide solution were purchased from Bio-Rad (Hercules, CA). [^{3}H]$PGF_{2\alpha}$ (212 Ci/mmol), [^{3}H]PGE_2 (159 Ci/mmol), *myo*-[2-^{3}H]inositol (18.0 Ci/mmol), cyclic AMP radioimmunoassay, and enhanced chemiluminescence kits were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). $PGF_{2\alpha}$ was obtained from Cayman Chemicals (Ann Arbor, MI). Cholera toxin and pertussis toxin were obtained from List Biological Laboratories Inc. (Campbell, CA). *N*-[2-([*p*-bromocinnamyl]amino)ethyl]-5-isoquinolinesulfonamide (H89 dihydrochloride) and dibutyryl cyclic AMP (dBcAMP) were obtained from Calbiochem (La Jolla, CA). 3-Isobutyl-1-methylxanthine (IBMX) was obtained from Sigma (St. Louis, MO) and 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid, disodium salt hydrate (DIDS) was purchased from Aldrich (Milwaukee, WI). Iloprost was purchased from Schering-Plough (Berlin, Germany). Anti $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_{12}$, and $G\alpha_{13}$ antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Transfection. The human FP was cloned in our laboratory (Kunapuli et al., 1997). A nine-amino-acid hemagglutinin epitope (HA) (YPYDVPDYA) was inserted between the N-terminal initiator methionine and the second amino acid, as described previously for the HA-IP (Smyth et al., 1996). The HA-FP cDNA was subcloned into the *Bam*HI/*Eco*RI sites of the mammalian expression vector pcDNA3.1 (Invitrogen) and used for stable transfection of HEK293 cells (HA-FP cells). The cDNA encoding hPGT, kindly donated by Victor L. Schuster of the Albert Einstein College of Medicine (Bronx, NY), was subcloned into the *Hin*dIII-*Not*I sites of pcDNA3.1 or pcDNA3.1/Zeo (Invitrogen). The hPGT in pcDNA3.1 was used for stable transfection of HEK293 cells (hPGT cells), whereas the hPGT in pcDNA3.1/Zeo was used for transfection of HA-FP cells. Subcloning into pcDNA3.1/Zeo allowed us to use zeocin as a second selection marker. Cells overexpressing both the HA-FP and the hPGT are indicated as HA-FP/hPGT cells. A $G\alpha_s$ dominant negative cDNA ($A^{366}S/G^{226}A/E^{268}A$) in pcDNAI was obtained from American Type Culture Collection, Manassas, VA). The minigene constructs in pcDNA3.1 were kindly donated by Drs. Annette Gilchrist and Heidi E. Hamm (Northwestern University, Chicago, IL).

HEK293 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, and 2 mM glutamine under 5% CO_2 at 37°C. For transfection, cells were seeded at 1.5×10^6 cells/100-mm dish and, the next day, transfected with 10 μg/dish cDNA by liposome-mediated transfer [*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate; Roche Molecular Biochemicals, Mannheim, Germany] according to the manufacturer's instructions. Eight hours after the beginning of transfection, the medium was changed and selection of the clones was performed with 0.75 mg/ml G418 (for HA-FP and hPGT cells) or with 0.75 mg/ml G418 plus 0.3 mg/ml zeocin (for HA-FP/hPGT cells). Binding of [^{3}H]$PGF_{2\alpha}$ to membranes from HA-FP/hPGT cells was saturable and revealed a B_{max} value of 0.52 pmol/mg protein and a K_d value of 17.6 nM. Nonspecific binding never exceeded 12% of total binding.

For transient transfection, cells were seeded at 3×10^5 cells/well in six-well plates and transfected 1 to 2 days later. When cells were transfected with two plasmids, we used 0.5 μg of each plasmid DNA mixed with 3 μl of FuGENE6 (Roche Molecular Biochemicals) per well in 2 ml of medium, according to the manufacturer's instructions. In the experiments with $G\alpha_s$ dominant negative or minigene constructs, three plasmids were used simultaneously. In these cases, we transfected each well with 0.4 μg of HA-FP or hPGT cDNA, and 1.2 μg of $G\alpha_s$ dominant negative or minigene constructs. Plasmid DNA was mixed with 4.5 μl of FuGENE6 per well in 2 ml of medium. Experiments were performed 48 h after transfection. NIH3T3 cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine under 10% CO_2 at 37°C.

Inositol Phosphate Formation. Inositol phosphate formation was measured in HA-FP/hPGT, hPGT, and NIH3T3 cells. Cells were plated in six-well plates at 2.5×10^5 cells/well and, the following day, incubated with 2 μCi/ml of *myo*-[2-^{3}H]inositol for 20 to 24 h in inositol-free DMEM. The day of the experiment, the medium was replaced with the same medium containing 20 mM LiCl, and the cells were stimulated with different concentrations of $PGF_{2\alpha}$. HA-FP/hPGT cells were stimulated for 10 min, whereas hPGT and NIH3T3 cells were stimulated for 30 min. Reactions were terminated by aspiration of the medium and inositol phosphates were extracted with 750 μl of 10 mM formic acid for 30 min, as described previously (Vezza et al., 1996; Habib et al., 1997). Agonist-stimulated inositol phosphate formation is expressed as a percentage of the vehicle-treated sample.

Uptake of [^{3}H]$PGF_{2\alpha}$. Uptake of [^{3}H]$PGF_{2\alpha}$ or [^{3}H]PGE_2 was carried out as described previously (Lu et al., 1996). HA-FP/hPGT and NIH3T3 cells were plated in six-well plates at 2.5×10^5 cells/well 1 day before the experiment. The hPGT cells did not adhere firmly to the wells. Thus, they were plated 3 to 4 days before the experiment, to allow them to adhere and spread.

Cells were washed with DMEM and incubated with 0.6 nM [^{3}H]$PGF_{2\alpha}$ or 0.7 nM [^{3}H]PGE_2 in the same medium at room temperature for different time intervals. Uptake was terminated by addition of ice-cold DMEM containing 5% bovine serum albumin. Cells were washed three times with DMEM, scraped in phosphate-buffered saline, and counted by liquid scintillation. In selected experiments, NIH3T3 cells were incubated for 15 min at 37°C with the anion transporter inhibitor DIDS (1 mM). Uptake of [^{3}H]$PGF_{2\alpha}$ was measured in the presence of DIDS, as described previously (Chan et al., 1998).

To assess whether stimulation of the FP alters hPGT activity, HA-FP/hPGT or hPGT cells were stimulated with different concentrations of $PGF_{2\alpha}$, or its vehicle, for 10 min at 37°C. When transiently transfected HEK293 cells were used, $PGF_{2\alpha}$ or iloprost was incubated for 30 min before measurement of [^{3}H]$PGF_{2\alpha}$ uptake. Cells were then washed and incubated with 0.6 nM [^{3}H]$PGF_{2\alpha}$ for 10 min at room temperature and uptake experiments were performed as described above. Results are expressed as a percentage of the vehicle-treated sample or as disintegrations per minute per microgram of protein. In the latter case, an aliquot was removed from each sample before addition of scintillation fluid. Protein concentration was determined using the Bradford assay with bovine serum albumin as a standard.

The cyclic AMP-dependent protein kinase inhibitor H89 and the cyclic AMP anolog dBcAMP (10 μM) were incubated for 30 min at 37°C before the addition of $PGF_{2\alpha}$ and the assessment of transporter activity.

Cholera or pertussis toxin was used at 250 ng/ml, unless otherwise

indicated in the text, and was incubated for 20 to 24 h at 37°C. Cells were then washed, stimulated with $PGF_{2\alpha}$ or its vehicle for 10 min at 37°C, washed again, and incubated with $[^3H]PGF_{2\alpha}$ to assess transporter activity.

Cyclic AMP Measurement. HA-FP/hPGT cells were plated in six-well plates at 2.5×10^5 cells/well. Cells were incubated with IBMX (0.5 mM) for 5 min and then stimulated with different concentrations of $PGF_{2\alpha}$, or its vehicle, for 10 min at 37°C. HA-FP cells were seeded at 1.2×10^5 cells/ml in 12-well plates and incubated, or not, with pertussis toxin 250 ng/ml for 20 to 24 h. Cells were then stimulated with different concentrations of $PGF_{2\alpha}$, or its vehicle, for 10 min in the presence of IBMX (0.5 mM). HA-IP cells (Smyth et al., 1996) were plated in 12-well plates at 1.2×10^5 cells/ml and incubated with cholera toxin for 20 to 24 h and stimulated with different concentrations of iloprost (10 pM-10 nM) for 10 min at 37°C.

Reactions were terminated by aspiration of the medium. Cyclic AMP was extracted with 600 μl of ice-cold 65% ethanol for 30 min and quantitated by radioimmunoassay, as described previously (Smyth et al., 1996).

Immunoblotting. A polyclonal peptide antibody was raised in rabbits to a 17 amino acid sequence of the carboxyl-terminal tail of the hPGT (H-RVKKNKEYNVQKAAGLI-OH) (Research Genetics Inc., Huntsville, AL). Immunoblotting was performed as described previously (Vezza et al., 1996). Briefly, blots were incubated for 1 h with the anti-hPGT antibody diluted 1:2000 in Tris-buffered saline (50 mM Tris-HCl, 250 mM NaCl, pH 7.4) containing 0.1% Tween 20 and 5% milk. Anti-$G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_{12}$, and $G\alpha_{13}$ antibodies were diluted 1:200 in the same buffer. A peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) was used as secondary antibody and was diluted 1:5000 in the same buffer. Positive bands were revealed by enhanced chemiluminescence.

Statistical Analysis. All results are presented as mean ± S.E.M. Statistical analysis was performed by Student's t test. A p value of 0.05 was considered to be statistically significant.

Results

We carried out functional experiments in NIH3T3 cells to determine whether the FP and the PGT might be coexpressed in the same cells. $PGF_{2\alpha}$ (1 nM-3 μM) induced a dose-dependent increase of inositol phosphate with an EC_{50} value of 52.5 ± 0.15 nM. The maximal response (4.09 ± 2.4-fold above the vehicle-treated sample, $n = 6$ experiments in duplicate), was attained at 1 μM $PGF_{2\alpha}$, demonstrating FP expression in NIH3T3 cells, consistent with previous data (Kunapuli et al., 1997). Expression of PGT in NIH3T3 cells was demonstrated measuring uptake of $[^3H]PGF_{2\alpha}$. Uptake was time-dependent and was inhibited by the anion transporter inhibitor DIDS (Fig. 1).

We developed HEK293 cells stably expressing the hemagglutinin-tagged FP (HA-FP) together with the hPGT (HA-FP/hPGT cells) to study the regulation of hPGT by FP. We also developed cell lines expressing the hPGT alone (hPGT cells) or the HA-FP alone (HA-FP cells). hPGT expression in several clones of HA-FP/hPGT and hPGT cells was verified by immunoblotting. Two clones were selected for further experiments and uptake of $[^3H]PGF_{2\alpha}$ in these clones confirmed hPGT expression. $[^3H]PGF_{2\alpha}$ uptake after 10-min incubation was 159.7 ± 13.0 dpm/μg of protein in HA-FP/hPGT cells, and 27.1 ± 2.4 dpm/μg of protein in hPGT cells; absolute dpm values ranged between 8,000 and 30,000 dpm/ml in HA-FP/hPGT cells, and between 700 and 1700 dpm/ml in hPGT cells.

Untransfected HEK293 and HA-FP cells did not take up PGs. In fact, the low level of radioactivity associated with untransfected cells or with HA-FP cells incubated with $[^3H]PGE_2$ was not diminished by incubation with DIDS. In addition, the radioactivity associated with HA-FP cells was similar to that associated with untransfected HEK293 cells. For example, the radioactivity measured in HEK293 after 20-min incubation with $[^3H]PGE_2$ was 0.57 and 0.45 dpm/μg of protein in the absence and in the presence of DIDS, respectively. In HA-FP cells, we measured 0.66 and 0.71 dpm/μg of protein in the absence and in the presence of DIDS, respectively. In contrast, $[^3H]PGE_2$ uptake at 10 min was inhibited by DIDS 1 mM by 93 to 96% in HA-FP/hPGT cells.

We checked FP expression in HA-FP/hPGT and in hPGT cells. As expected, HA-FP/hPGT cells express the FP, as demonstrated by an increase of inositol phosphate production upon stimulation with $PGF_{2\alpha}$ for 10 min (Fig. 2). By contrast, this response was absent in hPGT cells stimulated for up to 30 min with $PGF_{2\alpha}$ (Fig. 2).

We measured $[^3H]PGF_{2\alpha}$ uptake in HA-FP/hPGT cells to determine whether stimulation of the FP modifies hPGT activity. Cells were stimulated with different concentrations of $PGF_{2\alpha}$, washed, and incubated with $[^3H]PGF_{2\alpha}$ to measure uptake of this prostaglandin. Preincubation of the cells with $PGF_{2\alpha}$ caused a dose-dependent inhibition of $[^3H]PGF_{2\alpha}$ uptake (Fig. 3A). Similar results were obtained when using $[^3H]PGE_2$ in place of $[^3H]PGF_{2\alpha}$ to assess transporter activity (data not shown). Because residual nonradioactive $PGF_{2\alpha}$ could influence the uptake results, we measured the levels of $PGF_{2\alpha}$ in the washings by mass spectrometry. Surprisingly, we found residual, detectable $PGF_{2\alpha}$ even after washing the cells 10 times (7 and 3 nM after incubation of the cells with $PGF_{2\alpha}$ 100 nM and 1 μM, respectively). Although a dilutional effect by cold $PGF_{2\alpha}$ might have confounded our results and cannot be completely excluded, the high levels of $PGF_{2\alpha}$ after the first wash (15 and 202 nM after incubation with $PGF_{2\alpha}$ 100 nM and 1 μM, respectively) do not parallel the relatively small inhibition of uptake. In addition, the inhibitory effects of added $PGF_{2\alpha}$ on transport were similar after washing the

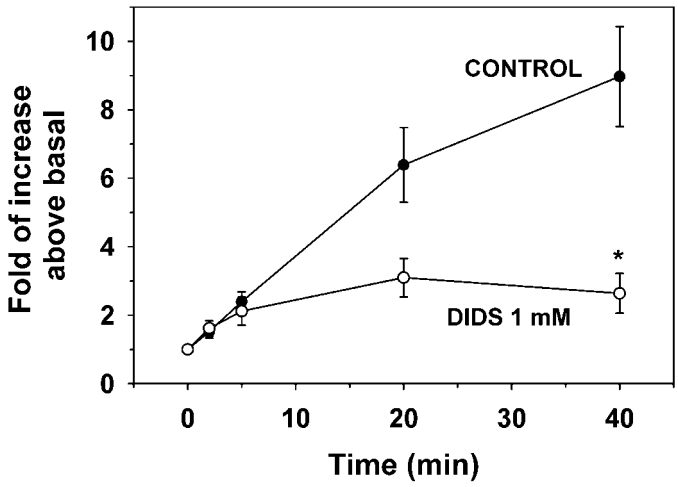

Fig. 1. NIH3T3 cells express PGT. Uptake of $[^3H]PGF_{2\alpha}$ was measured at different time intervals and was inhibited by preincubation of the cells for 15 min with the anion transporter inhibitor DIDS (1 mM). Results are expressed as fold of increase above basal (time zero) and are the mean ± S.E.M. of seven (in the absence of DIDS; control) and four (in the presence of DIDS) experiments performed in duplicate. *$p < 0.05$, compared with control.

cells once versus 10 times, despite a substantial difference in the residual concentrations of this compound at these time points.

We also evaluated uptake of [^{3}H]$PGF_{2\alpha}$ in HA-FP/hPGT cells pretreated with PGE_2. PGE_2 activates the FP with a potency roughly 50% lower than $PGF_{2\alpha}$, as demonstrated by measurement of inositol phosphate production in HA-FP/hPGT cells (4.4-fold increase over basal with 1 μM $PGF_{2\alpha}$ and 2.3-fold increase with 1 μM PGE_2). On the other hand, PGE_2 is imported by the hPGT with an affinity comparable with that of $PGF_{2\alpha}$ (Lu et al., 1996). Preincubation of HA-FP/hPGT cells with PGE_2 1 μM inhibited [^{3}H]$PGF_{2\alpha}$ uptake less efficiently than the same concentration of $PGF_{2\alpha}$ ([^{3}H]$PGF_{2\alpha}$ uptake: 75.5 $\pm$ 2.3% of vehicle-treated sample with PGE_2 versus 51.4 $\pm$ 1.3% with $PGF_{2\alpha}$, n = 3 experiments performed in duplicate), indicating that the regulation of the hPGT is receptor-mediated.

Inhibition of [^{3}H]$PGF_{2\alpha}$ uptake after stimulation of the FP with $PGF_{2\alpha}$ was also evident in NIH3T3 cells. These results were poorly reproducible (data not shown), possibly due to the low PGT expression. Indeed, we were able to detect PGT expression in NIH3T3 cells only by ribonuclease protection assay, but not by less sensitive techniques, such as Northern or Western blotting (data not shown).

Because the FP is a GPCR, we investigated the possibility of G protein-dependent regulation of the hPGT by the FP. Immunoblotting revealed that both HEK293 and NIH3T3 cells express at least $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_{12}$, and $G\alpha_{13}$ (data not shown). To assess whether $G\alpha_s$ or $G\alpha_i$ might be involved in hPGT regulation, we repeated uptake experiments in HA-FP/hPGT cells preincubated for 20 h with 250 ng/ml cholera or pertussis toxin. Cholera toxin completely abolished the inhibitory effect of $PGF_{2\alpha}$ whereas pertussis toxin, in parallel experiments, did not have any effect on the inhibition of transporter activity induced by stimulation of the cells with $PGF_{2\alpha}$ (Fig. 3A). The effect of cholera toxin on regulation of transporter activity was dose-dependent (Table 1) and concentrations higher than 5 ng/ml were necessary to counteract the FP-mediated inhibition of prostaglandin uptake. For example, dose-response curves to $PGF_{2\alpha}$ in the absence or in the presence of 1 ng/ml cholera toxin were superimposable (data not shown). To test the hypothesis that the effect of cholera toxin under our experimental conditions is due to $G\alpha_s$ down-regulation (Mochly-Rosen et al., 1988; Chang and Bourne, 1989; Boehm et al., 1996), we measured cyclic AMP levels in cells stably overexpressing the hemagglutinin-tagged PGI_2 receptor (HA-IP cells), which activates adenylate cyclase through $G\alpha_s$. Cyclic AMP levels increased from 0.01 $\pm$ 0.003 pmol/10^4 cells (n = 4) to 2.26 $\pm$ 0.14 pmol/10^4 cells (n = 4) in cells stimulated with 10 nM iloprost and not treated with cholera toxin. By contrast, cyclic AMP was not

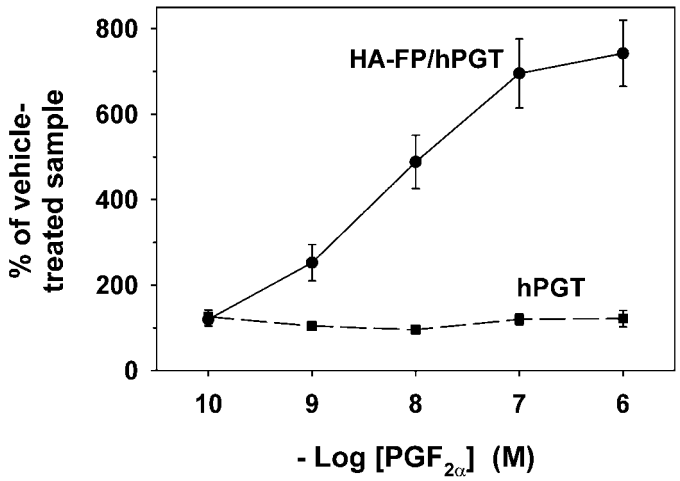

Fig. 2. HA-FP/hPGT, but not hPGT cells, express the FP. HEK293 cells were stably transfected either with the hPGT alone (hPGT cells) or with the hPGT together with the HA-FP (HA-FP/hPGT cells). Expression of the FP in HA-FP/hPGT cells was verified by measuring inositol phosphate production upon stimulation with different concentrations of $PGF_{2\alpha}$ for 10 min. No increase in inositol phosphate production was detected in hPGT cells incubated for 30 min with the same concentrations of $PGF_{2\alpha}$. Results are expressed as a percentage of the corresponding vehicle-treated sample and are the mean ± SEM of four (for HA-FP/hPGT cells) and five (for hPGT cells) experiments performed in duplicate.

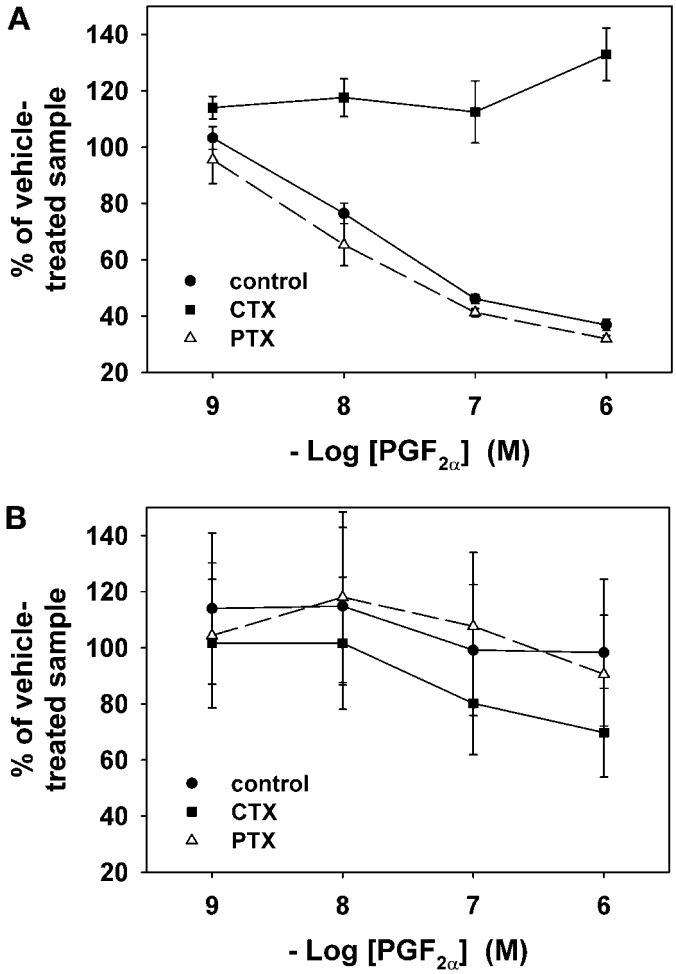

Fig. 3. A, stimulation of HA-FP/hPGT cells with $PGF_{2\alpha}$ inhibits [^{3}H]$PGF_{2\alpha}$ uptake. Cholera toxin (CTX), but not pertussis toxin (PTX), blocks the FP-dependent regulation of hPGT function. HA-FP/hPGT cells were incubated for 20 h with cholera or pertussis toxin (250 ng/ml) or left untreated (control). The following day, cells were washed, stimulated with different concentrations of $PGF_{2\alpha}$, or its vehicle, washed again, and incubated with [^{3}H]$PGF_{2\alpha}$ to assess uptake. Results are expressed as a percentage of the corresponding vehicle-treated sample and are the mean ± S.E.M. of five experiments performed in duplicate. B, stimulation of hPGT cells with $PGF_{2\alpha}$ does not inhibit [^{3}H]$PGF_{2\alpha}$ uptake. Cholera toxin (CTX) and pertussis toxin (PTX) do not have any effect. hPGT cells were incubated for 20 h with cholera or pertussis toxin (250 ng/ml) or left untreated (control) and experiments were carried out as described in A. Results are expressed as a percentage of the corresponding vehicle-treated sample and are the mean ± S.E.M. of four experiments performed in duplicate.

increased above the basal levels in cells pretreated with 250 ng/ml cholera toxin (0.01 ± 0.0006 pmol/10^4 unstimulated cells versus 0.02 ± 0.003 pmol/10^4 cells when stimulated with 10 nM iloprost, $n = 4$). We also measured cyclic AMP levels in HA-IP cells treated with different concentrations of cholera toxin (1–100 ng/ml) and stimulated with 0.1 nM iloprost. Even at 1 and 5 ng/ml, cholera toxin markedly inhibited the agonist-stimulated increase in cyclic AMP (from 69.1 ± 5.5-fold over basal in the absence of cholera toxin to 13.0 ± 0.7- and 10.0 ± 0.02-fold over basal after pretreatment with 1 and 5 ng/ml cholera toxin, respectively).

To determine whether ligation of the FP activates $G\alpha_s$, we measured cyclic AMP formation in HA-FP or HA-FP/hPGT cells stimulated for 10 min with different concentrations of $PGF_{2\alpha}$. Pretreatment of the cells for 5 min with 0.5 mM IBMX was necessary to detect a cyclic AMP increase under these experimental conditions. $PGF_{2\alpha}$, at concentrations that inhibit hPGT-mediated [^{3}H]$PGF_{2\alpha}$ uptake, increased cyclic AMP levels, irrespective of whether the cells had been pretreated with pertussis toxin. $PGF_{2\alpha}$ increased cyclic AMP levels 2.94 ± 0.43- and 3.21 ± 0.72-fold over basal ($n = 7$) at 100 nM and 1 μM, respectively, in HA-FP cells not pretreated with pertussis toxin (basal = 0.07 ± 0.006 pmol of cyclic AMP/10^4 cells). The increase in cyclic AMP levels was 3.31 ± 0.28 and 5.45 ± 0.62 fold over basal ($n = 9$) with $PGF_{2\alpha}$ 100 nM and 1 μM, respectively, in cells preincubated with 250 ng/ml pertussis toxin (basal = 0.06 ± 0.006 pmol of cyclic AMP/10^4 cells). An increase in cyclic AMP, even in the presence of pertussis toxin, which inhibits $G\alpha_i$, implies that $PGF_{2\alpha}$ activates $G\alpha_s$ to stimulate adenylate cyclase. Enhancement of receptor-mediated stimulation of cyclic AMP in pertussis toxin-treated cells or membrane preparations has already been reported (Hazeki and Ui, 1981; Katada et al., 1982).

We then investigated whether stimulation of the FP with its cognate ligand regulates hPGT through cyclic AMP-dependent protein kinase activation. HA-FP/hPGT cells were preincubated for 30 min with the stable cyclic AMP analog, dBcAMP, or with the cyclic AMP-dependent protein kinase inhibitor, H89. Neither stimulation nor kinase inhibition had any major effect on $PGF_{2\alpha}$-mediated inhibition of hPGT activity (Table 2). We verified the effectiveness of H89 as a cyclic AMP-dependent protein kinase inhibitor in in vitro phosphorylation experiments. At 10 μM, H89 completely suppressed histone H1 phosphorylation by cyclic AMP-dependent protein kinase (data not shown). In addition, we did not observe phosphorylation of hPGT in HA-FP/hPGT cells treated with dBcAMP or stimulated with $PGF_{2\alpha}$ (data not shown) indicating that hPGT regulation is not accompanied by cyclic AMP-dependent protein kinase-mediated phosphorylation and that this kinase does not play an important role in hPGT regulation.

In addition to cells expressing both HA-FP and hPGT, we developed a cell line expressing only the hPGT (hPGT cells) that was used as a negative control. Indeed, hPGT cells do not express the FP to a detectable extent, as reflected by a lack of stimulation of inositol phosphate synthesis by $PGF_{2\alpha}$ (Fig. 2). Thus, as expected, stimulation of hPGT cells with $PGF_{2\alpha}$ did not have any effect on hPGT activity, irrespective of pretreatment with cholera or pertussis toxin (Fig. 3B). In addition, neither H89 nor dBcAMP had any effect on [^{3}H]$PGF_{2\alpha}$ uptake in hPGT cells (data not shown).

We investigated whether FP stimulation with the isoprostane 8,12-*iso*-$iPF_{2\alpha}$-III would have effects similar to those of $PGF_{2\alpha}$ on hPGT regulation. This compound, previously known as 12-*iso*-$PGF_{2\alpha}$ (Rokach et al., 1997), also activates the FP (Kunapuli et al., 1997). 8,12-*iso*-$iPF_{2\alpha}$-III influenced [^{3}H]$PGF_{2\alpha}$ uptake only at 100 μM or above (Fig. 4), consistent with its lower potency as an FP ligand than $PGF_{2\alpha}$ (Kunapuli et al., 1997). 8,12-*iso*-$iPF_{2\alpha}$-III caused a modest increase in cyclic AMP production in HA-FP/hPGT cells at 1 and 10 μM (up to 2.35 ± 0.18 and 1.23 ± 0.17 pmol/well with 10 and 1 μM 8,12-*iso*-$iPF_{2\alpha}$-III, respectively, versus a basal level of 0.55 ± 0.17 pmol/well, $n = 3$) after 30-min incubation in the presence of IBMX. In parallel experiments, 1 μM $PGF_{2\alpha}$ increased cyclic AMP up to 3.03 ± 0.4 pmol/well ($n = 3$), again consistent with the isoprostane acting as a weak FP agonist. Measurement of inositol phosphate production in HA-FP/hPGT cells stimulated for 10 min with 8,12-*iso*-$iPF_{2\alpha}$-III confirmed that this isoprostane is roughly 100 times less potent than $PGF_{2\alpha}$ in activating the FP. Indeed, inositol phosphate production increased 2.7-fold over basal in cells stimulated with 1 μM $PGF_{2\alpha}$, and 2.1- and 2.6-fold over basal in cells stimulated with 10 and 100 μM 8,12-*iso*-$iPF_{2\alpha}$-III, respectively.

We studied HEK293 cells transiently transfected with hPGT and HA-FP to demonstrate that FP-mediated inhibition of hPGT function occurs in cells other than the particular HA-FP/hPGT clone that we selected for the majority of the experiments. Cells were stimulated with $PGF_{2\alpha}$ for 30 min before measurement of [^{3}H]$PGF_{2\alpha}$ uptake and $PGF_{2\alpha}$-dependent inhibition of the hPGT was again demonstrable (Fig. 5). In parallel experiments, we transiently transfected HEK293 cells with hPGT and HA-IP (Smyth et al., 1996). PGI_2, unlike $PGF_{2\alpha}$, is not transported via the PGT, although its receptor also couples to Gs activation (Coleman et al., 1994). HEK293 cells transfected with hPGT and HA-IP were stimulated with the PGI_2 analog iloprost, incubated for 30 min before mea-

TABLE 1

Low doses of cholera toxin do not counteract FP-mediated regulation of hPGT

HAFP/hPGT cells were incubated for 20 to 24 h with different concentrations of cholera toxin (CTX, 5–50 ng/ml) or left untreated. The following day, cells were washed, stimulated with $PGF_{2\alpha}$ 1 μM, or its vehicle, washed again, and incubated with [^{3}H]$PGF_{2\alpha}$ to assess uptake. Results are expressed as a percentage of the corresponding vehicle-treated sample and are the mean ± S.E.M. of three to five experiments performed in duplicate.

$PGF_{2\alpha}$	$PGF_{2\alpha}$+		
	CTX 5 ng/ml	CTX 25 ng/ml	CTX 50 ng/ml
45.30 ± 2.20	43.01 ± 4.15	65.71 ± 2.81	87.51 ± 3.92

TABLE 2

Cyclic AMP-dependent protein kinase does not play a major role in hPGT regulation

HA-FP/hPGT cells were incubated for 30 min with the kinase inhibitor H89 (10 μM) or with the cyclic AMP analog dBcAMP (10 μM). Control samples were treated with the vehicle. After 30 min, different concentrations of $PGF_{2\alpha}$ or its vehicle, were added and incubated for 10 min. Cells were then washed and incubated with [^{3}H]$PGF_{2\alpha}$ to assess uptake. Results are expressed as a percentage of the corresponding vehicle-treated sample and are the mean ± S.E.M. of five (for control and H89-treated cells) or four (for dBcAMP-treated cells) experiments performed in duplicate.

$PGF_{2\alpha}$	Control	H89	dBcAMP
1 nM	114.13 ± 5.33	110.13 ± 2.84	109.90 ± 3.26
10 nM	81.47 ± 5.76	91.51 ± 3.26	77.35 ± 4.95
100 nM	58.47 ± 2.05	54.60 ± 2.04	44.43 ± 2.61
1 μM	47.30 ± 3.2	48.26 ± 2.68	34.33 ± 2.16

surement of [^{3}H]$PGF_{2\alpha}$ uptake. Iloprost did not have any effect on hPGT activity under these conditions (Fig. 5), despite causing a marked increase in cyclic AMP levels (up to 354.2 ± 47.4 and 235.0 ± 12.5 pmol/well with 100 and 1 nM iloprost, respectively, versus a basal level of 3.77 ± 0.29 pmol/well, $n = 3$).

We used a $G\alpha_s$ dominant negative construct (Iiri et al., 1999) and a $G\alpha_s$ minigene construct (Rasenick et al., 1994; Gilchrist et al., 1999) to implicate further $G\alpha_s$ in FP-mediated hPGT regulation. The former has been shown to inhibit human chorionic gonadotropin-stimulated cyclic AMP accumulation in transfected COS7 cells (Iiri et al., 1999), whereas the latter inhibits β-adrenergic activation of $G\alpha_s$ (Rasenick et al., 1994). HEK293 cells were transiently transfected with HA-FP, hPGT, and the construct of interest. Cells were stimulated 2 days later with $PGF_{2\alpha}$ and incubated with [^{3}H]$PGF_{2\alpha}$ to evaluate transporter activity.

Although the Gs dominant negative was not expressed at high levels, as determined by immunoblotting, transfection of cells with this construct (Fig. 6) or the $G\alpha_s$ minigene (Fig. 7) blunted the inhibition of transporter activity after FP stimulation. On the other hand, expression of $G\alpha_i$ or $G\alpha_i$ random order minigene constructs failed to influence FP-mediated inhibition of transporter activity (Fig. 7).

Discussion

Prostaglandins are charged organic anions at physiological pH, thus they may transverse biological membranes inefficiently (Bito and Baroody, 1975; Kanai et al., 1995; Lu et al., 1996). Although initial hydrolysis and subsequent oxidation rapidly inactivate both PGI2 and thromboxane A_2, PGE_2, PGD_2, and $PGF_{2\alpha}$ are metabolized intracellularly. Thus, it is assumed that termination of the activity of these PGs in vivo is due to uptake followed by intracellular oxidation (Schuster, 1998). Although a carrier-mediated transport for PGs has been hypothesized since the 1960s and multidrug resistance proteins that are transporters for leukotrienes have been characterized (Cole and Deeley, 1998), a PGT has been cloned only recently (Kanai et al., 1995; Lu et al., 1996). The PGT mediates both the uptake (Lu et al., 1996) and the efflux (Chan et al., 1998) of PGs, thus it may be important both in termination of PG effects, and in extracellular release of newly synthesized PGs for ligation of membrane GPCRs.

Untransfected HEK293 cells and cells transfected only with the HA-FP failed to take up [^{3}H]$PGF_{2\alpha}$. This indicates that expression of the FP per se does not influence [^{3}H]$PGF_{2\alpha}$ uptake, and that possible internalization of the ligand-bound receptor does not contribute significantly to the intracellular

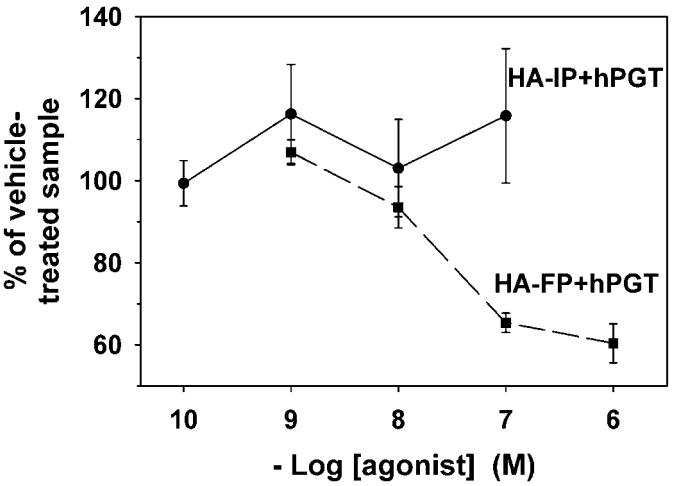

Fig. 5. Prostacyclin receptor (HA-IP) does not regulate [^{3}H]$PGF_{2\alpha}$ uptake. HEK293 cells were transiently transfected with hPGT together with HA-IP (HA-IP + hPGT) or with HA-FP (HA-FP + hPGT). Two days after transfection, cells were stimulated with different concentrations of iloprost or $PGF_{2\alpha}$, respectively, or their vehicle. Results are expressed as a percentage of the corresponding vehicle-treated samples and are the mean ± S.E.M. of five experiments performed in duplicate.

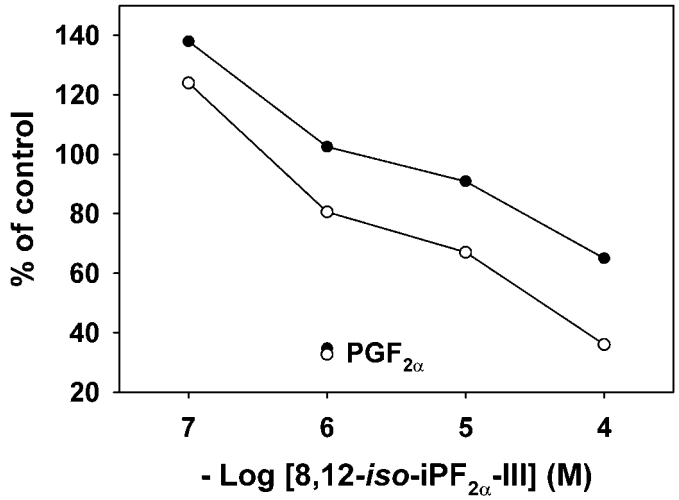

Fig. 4. Stimulation of HA-FP/hPGT cells with 8,12-*iso*-$iPF_{2\alpha}$-III slightly inhibits [^{3}H]$PGF_{2\alpha}$ or [^{3}H]PGE_2 uptake. HA-FP/hPGT cells were stimulated with 8,12-*iso*-$iPF_{2\alpha}$-III, or its vehicle, for 10 (closed symbols) or 30 min (open symbols). Cells were washed and incubated with [^{3}H]$PGF_{2\alpha}$ or [^{3}H]PGE_2 for 10 min to assess uptake. Similar results were obtained when using [^{3}H]$PGF_{2\alpha}$ or [^{3}H]PGE_2. Results are expressed as a percentage of the corresponding vehicle-treated sample and are from one experiment performed in duplicate, representative of two to seven experiments. $PGF_{2\alpha}$ (1 μM) was tested in parallel experiments as a positive control.

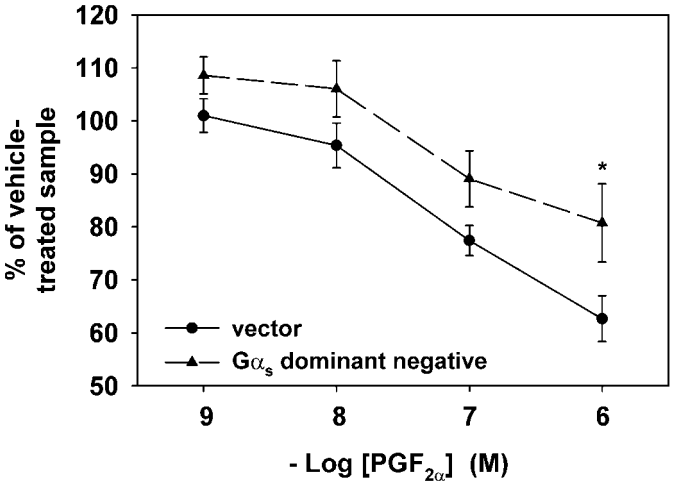

Fig. 6. FP-mediated inhibition of [^{3}H]$PGF_{2\alpha}$ uptake is blunted by expression of a $G\alpha_s$ dominant negative construct. HEK293 cells were transiently transfected with hPGT, HA-FP, and either the empty vector or a $G\alpha_s$ dominant negative construct, as described under *Experimental Procedures*. Two days after transfection, cells were stimulated with different concentrations of $PGF_{2\alpha}$. Results are expressed as a percentage of the corresponding vehicle-treated samples and are the mean ± S.E.M. of five (when using 1 and 10 nM $PGF_{2\alpha}$) or nine (when using 100 nM or 1 μM $PGF_{2\alpha}$) experiments performed in duplicate. *$p = 0.05$, compared with vector-transfected cells.

levels of [^{3}H]$PGF_{2\alpha}$. On the other hand, engagement of the receptor with its cognate ligand dose-dependently regulated import of both $PGF_{2\alpha}$ and PGE_2 when the hPGT was present.

Given that cold $PGF_{2\alpha}$ is not completely removed by washing the cells before the incubation with [^{3}H]$PGF_{2\alpha}$, we cannot completely exclude a dilutional effect of the residual cold ligand. On the other hand, the amount of residual $PGF_{2\alpha}$ does not correlate with inhibition of uptake, suggesting that a receptor-mediated effect on transporter activity does indeed exist. This hypothesis is strengthened by the following observations: $PGF_{2\alpha}$ did not influence transporter activity in hPGT cells, which do not express the HA-FP; and PGE_2 regulates the hPGT in HA-FP/hPGT cells with a potency lower than $PGF_{2\alpha}$, consistent with the lower potency of PGE_2 as an FP agonist.

Activation of the FP by a structurally distinct ligand, the isoprostane 8,12-*iso*-i$PF_{2\alpha}$-III, also regulated transporter function. Isoprostanes are free radical-catalyzed products of arachidonic acid and may act as incidental ligands at membrane receptors for prostanoids in vivo (Audoly et al., 2000). Although the isoprostane is a less potent FP agonist, both $PGF_{2\alpha}$ and 8,12-*iso*-i$PF_{2\alpha}$-III activate phospholipase C and adenylate cyclase, presumably via Gq and Gs, respectively. The two ligands can also activate divergent signaling pathways, at least in cardiac myocytes (Kunapuli et al., 1998).

To address the role of G proteins in receptor-transporter interactions, we first examined the effects of pertussis and cholera toxin, probes for Gi and Gs, respectively. Pertussis toxin ADP-ribosylates the α subunit of G proteins of the Gi family when the α subunit is bound to GDP. Thus, pertussis toxin stabilizes $G\alpha_i$ in the inactive conformation. For this reason, pertussis toxin is widely used as an inhibitor of $G\alpha_i$ (Simon et al., 1991). Regulation of $G\alpha_s$ by cholera toxin is complex. ADP ribosylation of $G\alpha_s$ by cholera toxin stabilizes the GTP-bound conformation of $G\alpha_s$ and decreases its intrinsic GTPase activity (Casey and Gilman, 1988; Freissmuth et al., 1989). This leads to a persistent activation of this G protein, thereby enhancing adenylate cyclase stimulation and production of intracellular cyclic AMP. Prolonged incubation of the cells with cholera toxin, on the other hand, results in down-regulation of $G\alpha_s$ with loss of this G protein from the plasma membrane (Mochly-Rosen et al., 1988; Chang and Bourne, 1989; Boehm et al., 1996). When used to probe FP-dependent pathways, cholera toxin, but not pertussis toxin, blocked the effect of FP activation by $PGF_{2\alpha}$ on transporter function in a dose-dependent manner. These results suggest involvement of Gs in the interaction of the FP with the transporter. Prolonged incubation of cells with cholera toxin has been described to cause $G\alpha_s$ down-regulation (Mochly-Rosen et al., 1988; Chang and Bourne, 1989; Boehm et al., 1996) and, as a consequence, reduced hormone-stimulated cyclic AMP production (Mochly-Rosen et al., 1988). Consistent with this, we observed that the PGI_2 analog iloprost is less efficacious in stimulating an increase in cyclic AMP in HA-IP cells treated for 20 to 24 h with cholera toxin. Although cholera toxin, at concentrations as low as 1 ng/ml, markedly decreased iloprost-stimulated cyclic AMP, doses of the toxin higher than 5 ng/ml were necessary to counteract the effect of $PGF_{2\alpha}$ on [^{3}H]$PGF_{2\alpha}$ uptake in HA-FP/hPGT cells. Although cholera toxin may have effects unrelated to $G\alpha_s$, these results distinguish dose-dependent regulation of adenylate cyclase from that of the hPGT. Indeed, interaction of the FP with the transporter seems to be largely independent of its ability to catalyze adenylate cyclase activation. Neither pharmacological activation nor inhibition of cyclic AMP-dependent protein kinase modified FP-mediated regulation of transporter function. In addition, marked activation of the cyclase via Gs coupled to the PGI_2 receptor also failed to regulate transporter function. Finally, ligation of the FP with $PGF_{2\alpha}$ fails to result in phosphorylation of the transporter, despite regulation of its function.

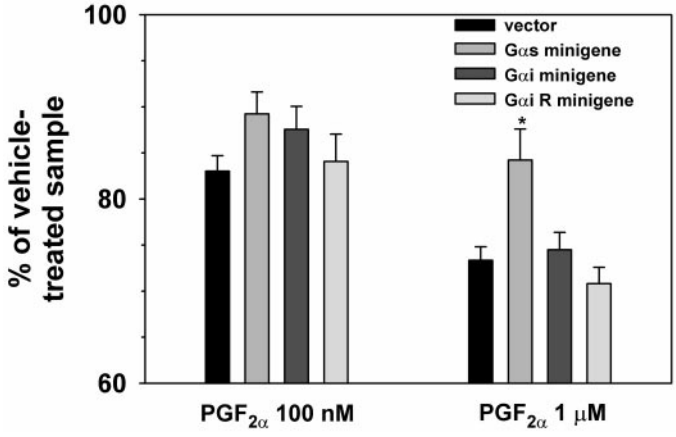

Fig. 7. FP-mediated inhibition of [^{3}H]$PGF_{2\alpha}$ uptake is blunted by expression of $G\alpha_s$, but not of $G\alpha_i$ or $G\alpha_i$ in random order ($G\alpha_i$ R) minigene constructs. HEK293 cells were transiently transfected with hPGT, HA-FP, and either the empty vector or minigene constructs, as described under *Experimental Procedures*. Two days after transfection, cells were stimulated with 100 nM and 1 μM $PGF_{2\alpha}$. Results are expressed as a percentage of the corresponding vehicle-treated samples and are the mean ± S.E.M. of six to eight experiments performed in duplicate. *p = 0.011, compared with vector-transfected cells.

Although these observations do not support a role for Gs-mediated cyclase activation in the regulation of FP-dependent inhibition of transporter function, additional experiments do support the importance of Gs in this phenomenon. Others have shown that cholera toxin causes persistent activation of $G\alpha_s$ by inhibiting its intrinsic GTPase activity (Casey and Gilman, 1988; Freissmuth et al., 1989); thereafter, it causes a loss of $G\alpha_s$ from the plasma membrane (Mochly-Rosen et al., 1988; Chang and Bourne, 1989; Boehm et al., 1996). Thus, the effects of the toxin might be attributable to adenylate cyclase activation, excluded for reasons mentioned above, or be due to $G\alpha_s$ down-regulation. Results obtained by specific inhibition of $G\alpha_s$ with dominant negative constructs support the latter contention. By contrast, a minigene construct directed against $G\alpha_i$ failed to modify FP-regulated hPGT function, as did pertussis toxin. Although the mechanism through which FP-mediated Gs activation regulates hPGT functions is presently unknown, the kinetics of the reaction suggest that a protein-protein interaction might be responsible for hPGT regulation. Additional experiments would be required to determine whether Gs interacts directly with hPGT, or whether other proteins mediate this interaction.

In conclusion, engagement of the FP by its cognate ligand $PGF_{2\alpha}$ and, to a much lesser extent, by the isoprostane 8,12-*iso*-i$PF_{2\alpha}$-III dose-dependently inhibits import of $PGF_{2\alpha}$, potentially for metabolic inactivation. Should this system operate in vivo, it might serve initially to amplify rapidly the effects of high local concentrations of $PGF_{2\alpha}$, which would subsequently result in receptor desensitization. Prostanoids

have been shown to augment more gradually their formation by induction of cyclooxygenase-2 (Barry et al., 1997). Although the details by which receptor activation regulates transporter function remain to be elucidated, our data suggest that interaction of the receptor with the transporter is dependent on $G\alpha_s$, apparently functioning in a role independent of its capacity to catalyze activation of adenylate cyclase.

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