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Regulation of prostaglandin EP_1 and EP_4 receptor signaling by carrier-mediated ligand reuptake

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Abstract

After synthesis and release from cells, prostaglandin E_2 (PGE_2) undergoes reuptake by the prostaglandin transporter (PGT), followed by cytoplasmic oxidation. Although genetic inactivation of PGT in mice and humans results in distinctive phenotypes, and although experiments in localized environments show that manipulating PGT alters downstream cellular events, a direct mechanistic link between PGT activity and PGE_2 (EP) receptor activation has not been made. Toward this end, we created two reconstituted systems to examine the effect of PGT expression on PGE_2 signaling via two of its receptors (EP_1 and EP_4). In human embryonic kidney cells engineered to express the EP_1 receptor, exogenous PGE_2 induced a dose-dependent increase in cytoplasmic Ca^{2+}. When PGT was expressed at the plasma membrane, the PGE_2 dose–response curve was right-shifted, consistent with reduction in cell surface PGE_2 availability; a potent PGT inhibitor acutely reversed this shift. When bradykinin was used to induce endogenous PGE_2 release, PGT expression similarly induced a reduction in Ca^{2+} responses. In separate experiments using Madin–Darby Canine Kidney cells engineered to express the PGE_2 receptor EP_4, bradykinin again induced autocrine PGE_2 signaling, as judged by an abrupt increase in intracellular cAMP. As in the EP_1 experiments, expression of PGT at the plasma membrane caused a reduction in bradykinin-induced cAMP accumulation. Pharmacological concentrations of exogenous PGE_2 induced EP_4 receptor desensitization, an effect that was mitigated by PGT. Thus, at an autocrine/paracrine level, plasma membrane PGT regulates PGE_2 signaling by decreasing ligand availability at cell surface receptors.

Abbreviations

DMEM, Dulbecco's modified Eagle's medium; EP_1-HEK, HEK cells stably expressing the PGE_2 receptor EP_1; EP, prostaglandin E_2 receptor; FBS, fetal bovine serum; Fura-2 AM, fura-2 acetoxymethyl ester; G418, geneticin; GAPDH, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescence protein; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; HPGD, 15-hydroxyprostaglandin dehydrogenase; HRP, horseradish peroxidase; MDCK, Madin–Darby Canine Kidney; P/S, penicillin/streptomycin; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; $PGF_2\alpha$, prostaglanding $F_2\alpha$; PG, prostaglandin; PGT-EP_1-HEK, HEK cells stably expressing both the PGE_2 receptor EP_1 and the prostaglandin transporter PGT; PGT-GFP-MDCK, MDCK cells stably expressing GFP-tagged PGT; PGT, prostaglandin transporter; PSF, phenylmethyl sulfonyl fluoride; SLC, solute carrier; T26A, *N*-(2-(2-(2-azidoethoxy)ethoxy)

ethyl)-4-((4-((2-(2-(2-benzamidoethoxy) ethoxy)ethyl)amino)-6-((4-hydroxyphenyl) amino)-1,3,5-triazin-2-yl)amino)benzamide; WT-HEK, wild-type HEK cell line.

Introduction

In recent years, mechanisms mediating prostaglandin E_2 (PGE_2) signal termination have become increasingly clear. Following synthesis, release, and binding to its cognate receptors ($EP_{1\text{-}4}$), PGE_2 is metabolically inactivated by a two-step process, consisting first of energetically active uptake across the plasma membrane by the prostaglandin transporter (PGT) (SLCO2A1) (Kanai et al. 1995), and then cytoplasmic oxidative inactivation by 15-OH prostaglandin dehydrogenase (HPGD) (Tai et al. 2002). PGE_2 influx across the plasma membrane by PGT is rate limiting for this inactivation, because heterologous expression of HPGD without PGT is insufficient to result in PGE_2 oxidation (Nomura et al. 2004).

Based on this model, our laboratory has advanced the hypothesis that PGE_2 signaling is akin to synaptic signaling (Nomura et al. 2005). Notably, both neurotransmitters and prostanoids are synthesized by inducible enzymes (Saadat et al. 1989; Smith et al. 2000; Stichtenoth et al. 2001; Murakami and Kudo 2004); both systems involve triggered release of ligand into the extracellular compartment (Greengard 2001; Kudo and Murakami 2002); both sets of G-protein coupled receptors (GPCRs) utilize similar molecular signaling and regulatory mechanisms (Lefkowitz 1993; Neuschafer-Rube et al. 2004); and both sets of ligands undergo reuptake by plasma membrane carriers that are located on the ligand-releasing cell (Kanai et al. 1995; Bao et al. 2002; Nomura et al. 2005; Gether et al. 2006; Kristensen et al. 2011).

Despite the attractiveness of this hypothesis, it has not been shown definitively that PGE_2 reuptake by PGT can control signaling at a local level. Although genetic inactivation of PGT in mice results in patent ductus arteriosus (Chang et al. 2010), and humans who are homozygous null at the PGT locus have pachydermoperiostosis (Busch et al. 2012; Diggle et al. 2012; Sasaki et al. 2012; Seifert et al. 2012; Zhang et al. 2012), in both cases systemic levels of PGE_2 are increased, and ancillary studies suggest that these phenotypes might result from elevated circulating PGE_2 acting as a hormone (Ueda et al. 1980; Ringel et al. 1982; Drvaric et al. 1989; Cattral et al. 1994; Letts et al. 1994; Reese et al. 2000). Moreover, although experiments using localized (autocrine/paracrine) model systems have been interpreted to indicate that PGT alters PGE_2 signaling, the biological events measured in these studies were considerably distal to the EP receptors (Henry et al. 2005; Gordon et al. 2008; Subbaramaiah et al. 2011). Thus, a direct mechanistic link between cell surface PGT activity and EP receptor activation has not been made.

Here, we coexpressed either of two representative PGE_2 ("EP") receptors, along with the prostaglandin reuptake carrier PGT, in kidney cell lines. We then acutely manipulated cell surface PGT function and directly measured either signaling through the receptors or ligand-induced receptor desensitization. Our results demonstrate that plasma membrane PGT modulates the concentration of cell surface PGE_2 in an autocrine/paracrine signaling mode. As a result, PGT directly modulates ligand access to, and activation of, EP_1 and EP_4 receptors.

Materials and Methods

Cell lines

Wild-type human embryonic kidney-293 cells (WT-HEK), wild-type Madin–Darby Canine Kidney (WT-MDCK) cells, and MDCK cells expressing green fluorescence protein-tagged PGT (PGT-GFP-MDCK) (Endo et al. 2002) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HEK cells stably expressing the EP_1 receptor (EP_1-HEK) (Peti-Peterdi et al. 2003) were cultured in F12 medium supplemented with 10% FBS, 1% P/S, 1.2 g/L $NaHCO_3$, and 0.05 mg/mL G418. We generated a derivative of the EP_1-HEK line stably expressing PGT (PGT-EP_1-HEK) by transfecting EP_1-HEK cells with human PGT cloned into the pcDNA3.1/Hygro (+) vector (Invitrogen, Grand Island, NY), followed by selection with hygromycin (0.25 mg/mL) in F12 media as described for EP_1-HEK.

mRNA quantification

EP_1, PGT, and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNAs in EP_1-HEK cells and in PGT-EP_1-HEK cells were quantified by qRT-PCR using the SYBR system (Invitrogen). The EP_1 (mouse) primers used were: 5′-CATCCGCTAGGCTCAGGTTA-3′ (forward) and 5′-AGCAGGAGCCAAGTTCCAG-3′ (backward). The PGT (human) primers used were: 5′-TGTACAGGAGTTGGCAGAGC-3′ (forward) and 5′-AGCGACACCTCTACTAGCCG-3′ (backward). The GAPDH (human) primers used were: 5′-AATGAAGGGGTCATTGATGG-3′ (forward) 5′-AAGGTGAAGGTCGGAGTCAA-3′ (backward).

Measurement of PGT function

PGT-mediated ^{3}H-PGE_2 uptake was measured using methods previously described (Kanai et al. 1995; Chi et al. 2006).

Protein quantification by Western blotting

Protein extraction and quantification by Western blotting were conducted using methods previously described (Syeda et al. 2012). EP_1 was probed with an anti-EP_1 polyclonal antibody (Cayman Chemical, Ann Arbor, MI) overnight at 4°C, with a goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody as secondary antibody.

Measurements of intracellular Ca^{2+} transients

Changes in cytosolic Ca^{2+} levels were measured in cells loaded with the ratiometric Ca^{2+} indicator Fura-2 AM (Molecular Probes, Grand Island, NY) and imaged on an epifluorescence microscope as previously described (Suadicani et al. 2004, 2006). Experiments were performed with cells bathed in Tyrode solution ([in mmol/L] 137.0 NaCl, 2.7 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 12.0 $NaHCO_3$, 0.5 NaH_2PO_4, 5.5 glucose, and 5 HEPES; pH 7.4). Values of intracellular Ca^{2+} levels were obtained from Fura-2 ratio images using an in vitro calibration curve.

EP_4 cell surface expression

WT-MDCK and PGT-GFP-MDCK cells were transiently transfected with a human EP_4-expressing cDNA (UMR cDNA Resource Center, www.cdna.org) using lipofectamine reagent (Invitrogen) 48 h after being seeded. At 24 h after transfection, the cells were treated with exogenous PGE_2 so as to obtain immediate medium PGE_2 concentrations of 0, 10, 20, 30, or 50 nmol/L. These experiments were performed in the absence or presence of the PGT inhibitor T26A (Chi et al. 2011) (5 μmol/L) at 37°C for 10 min. Immediately before harvesting the cells, media were collected to determine the result of contact with the cell monolayers on extracellular PGE_2 concentrations. Cells were then collected and lysed with buffer containing 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 10 mmol/L $MgCl_2$, 0.1 mmol/L PSF, and 20 μmol/L indomethacin to inhibit any further PGE_2 synthesis. Cell surface EP_4 receptor expression was quantified by [^{3}H]-PGE_2 binding as previously described (Nishigaki et al. 1996). Briefly, the harvested cells were homogenized using a Potter–Elvehjem homogenizer in an ice-cold lysing buffer. The homogenate was centrifuged at 800 rpm for 5 min, and the supernatant was further centrifuged at 19,500 rpm for 45 min at 4°C. The pellet was washed once and then resuspended in 100 μL buffer A (20 mmol/L HEPES-NaOH, pH 7.4, 1 mmol/L EDTA, 10 mmol/L $MgCl_2$) containing 4 nmol/L [^{3}H]-PGE_2 (170,000 dpm) and incubated at 30°C for 1 h. One milliliter cold buffer A was then added to stop binding. The mixture was filtered through a glass fiber which was then transferred to a scintillation vial. Ten milliliter scintillation solution was added to the vial and [^{3}H]-PGE_2 was counted by scintillation counting. Nonspecific binding was determined using a 1000-fold excess of unlabeled PGE_2 in the incubation mixture. The specific binding was calculated by subtracting the nonspecific binding value from the total binding value.

PGE_2 measurement

Extracellular PGE_2 concentrations were quantified using an enzyme-linked immunoassay kit (Cayman Chemical) according to the manufacturer's protocol.

cAMP measurement

WT-MDCK and PGT-GFP-MDCK cells were transfected with the EP_4-expressing vector 24 h after being seeded onto six-well plates. Twenty-four hours after transfection, they were incubated with the cAMP/cGMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 250 μmol/L) for an additional 18 h. Cells were then stimulated either with 10 μmol/L bradykinin or with various concentrations of exogenous PGE_2 (0, 10, 20, 30, or 50 nmol/L), in the absence or presence of T26A (5 μmol/L), at 37°C for 30 and/or 10 min, respectively. Thereafter, cells were washed twice with phosphate-buffered saline (PBS) and were lysed with PBS containing 0.1 mol/L HCl and 0.1% triton-X 100 (250 μL/well) at room temperature for 15 min. Cell lysates were centrifuged (10,000*g*, 4°C for 10 min), and cAMP in the supernatants was measured using an enzyme-linked immunoassay (ELISA) kit from Cayman Chemical according to the manufacturer's protocol.

Results

Generation of a cell line coexpressing PGT and EP_1

To investigate functional interplay between the PGT transporter and EP_1 receptors, we began with a cell line that stably overexpresses the mouse EP_1 receptor, "EP_1-HEK" (Peti-Peterdi et al. 2003). From this, we generated a new cell line, "PGT-EP_1-HEK," which stably overexpresses both mouse EP_1 and human PGT. Figure 1A shows that PGT mRNA expression in PGT-EP_1-HEK cells is about 140-fold that of EP_1-HEK cells. Figure 1B shows that PGT-EP_1-HEK cells exhibit significant tracer PGE_2 uptake, whereas EP_1-HEK cells exhibit almost no PGE_2 uptake. Figure 1C and D show that EP_1 mRNA and protein expression in PGT-EP_1-HEK cells were not different from that in the original EP_1-HEK cells.

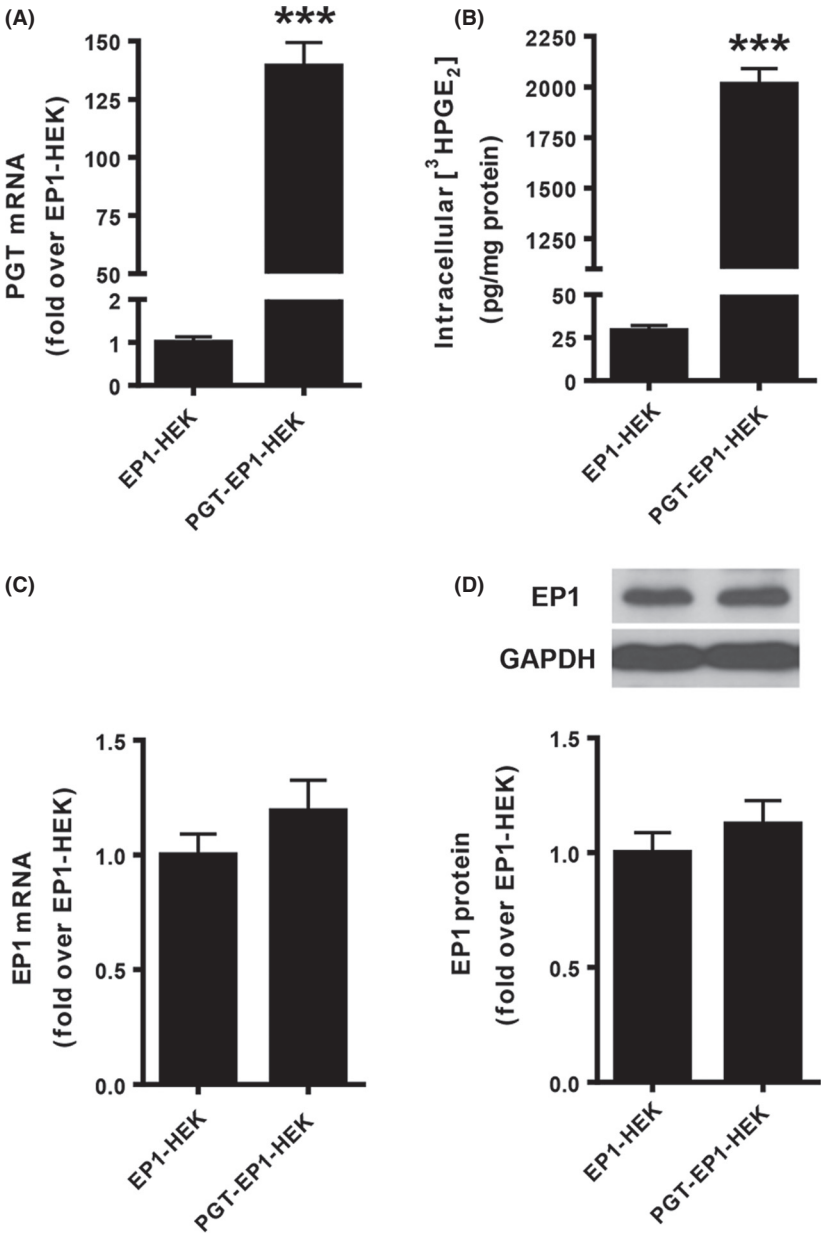

Figure 1. Properties of EP_1-HEK and PGT-EP_1-HEK cell lines. (A and C) PGT and EP_1 mRNA expression levels in EP_1-HEK and PGT-EP_1-HEK cell lines. Cells were seeded onto six-well plates and harvested 72 h after seeding. Total RNA was extracted and mRNA was determined by qRT-PCR. PGT-EP_1-HEK cells expressed ~140-fold more PGT mRNA than EP_1-HEK cells, whereas EP_1-HEK and PGT-EP_1-HEK cells expressed comparable levels of EP_1 mRNA. (B) ^{3}H-PGE_2 uptake in EP_1-HEK and PGT-EP_1-HEK cell lines. Cells were seeded onto 24-well plates and ^{3}H-PGE_2 uptake was measured 72 h after seeding. PGT-expressing cells exhibited 60- to 70-fold more ^{3}H-PGE_2 uptake than EP_1-HEK control cells. (D) EP_1 protein expression levels in EP_1-HEK and PGT-EP_1-HEK cell lines. Cells were seeded onto 10-cm plates and harvested 72 h after seeding. Protein was extracted and quantified by Western blot. Upper panel shows a representative immunoblot; lower panel shows quantitation of three blots. EP_1-HEK and PGT-EP_1-HEK cells exhibited no difference in EP_1 protein expression. Values are mean ± SEM ($n = 3$ for each experiment). ***$P < 0.0001$ by *t*-test.

PGT modulates EP_1-mediated Ca^{2+} release in response to exogenously applied PGE_2

EP_1 receptor activation triggers intracellular Ca^{2+} release (Sugimoto and Narumiya 2007). Indeed, in both EP_1-HEK and PGT-EP_1-HEK cells, activation of EP_1 by exogenous PGE_2 (0.3 nmol/L to 3 μmol/L) induced a concentration-dependent rise in intracellular Ca^{2+} levels (Fig. 2A). However, in PGT-EP_1-HEK cells, which express the PGT reuptake carrier, the dose–response curve for

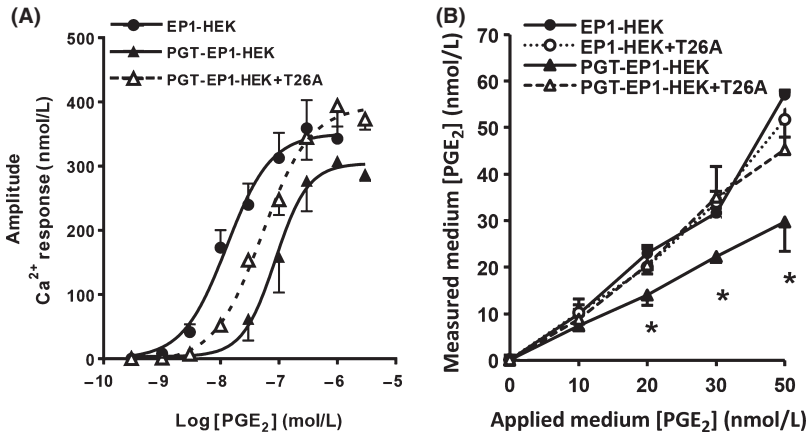

Figure 2. PGT modulates PGE_2-induced Ca^{2+} responses and medium PGE_2 levels. (A) Dose-dependent responses of the increase in intracellular Ca^{2+} concentration induced by PGE_2 stimulation of EP_1-HEK (n = 37 cells), and of PGT-EP_1-HEK cells in the absence (n = 211 cells) and presence of the PGT blocker T26A (5 μmol/L, n = 340 cells). PGT shifted the PGE_2/Ca^{2+} dose–response curve to the right; PGT inhibition by T26A reversed this effect (see text for statistical analyses). (B) Final PGE_2 concentration in media following 10 min exposure to EP_1-HEK or PGT-EP_1-HEK monolayers in the presence and absence of T26A (5 μmol/L). PGT expression lowered the medium PGE_2 concentration. Values are mean ± SEM (n = 3 for each experiment). *P < 0.05.

PGE_2 was significantly right-shifted (Fig. 2A). The EC_{50} (half-maximal effective concentration) value for PGE_2 in EP_1-HEK cells was 12.8 nmol/L (95% CI = 7.2–22.8 nmol/L, n = 37 cells), whereas in PGT-EP_1-HEK cells it was 86.9 nmol/L (95% CI = 51.7–146.0 nmol/L, n = 211 cells) (P < 0.01). Of note, this rightward shift of the PGE_2 dose–response curve cannot be attributed to differing levels of EP_1 expression (see Fig. 1C and D). The maximal response to PGE_2 was slightly but not significantly lower in PGT-EP_1-HEK compared to EP_1-HEK cells (Fig. 2A).

Acute inhibition of PGT transport in PGT-EP_1-HEK cells with T26A (5 μmol/L) resulted in significantly higher maximal Ca^{2+} responses to PGE_2 (P < 0.05) compared to nontreated PGT-EP_1-HEK cells (Fig. 2A). T26A shifted the PGE_2 dose–response curve directionally, albeit not statistically significantly, back toward that of EP1-HEK cells (EC_{50} = 51.8 nmol/L; 95% CI = 36.3–73.9 nmol/L, n = 324). Given the known effect of PGT to mediate PGE_2 uptake from the extracellular compartment (Nomura et al. 2004), the data of Figure 2A suggest that, at any given concentration of PGE_2 added to the culture medium, plasma membrane PGT lowers the PGE_2 concentration at the cell surface, as revealed by a reduction in EP_1 receptor activation.

To test this hypothesis further, we added exogenous PGE_2 to EP_1-HEK and PGT-EP_1-HEK cell monolayers so as to achieve initial PGE_2 concentrations of 0, 10, 20, 30, and 50 nmol/L. We then measured PGE_2 concentrations in the bathing media after 10 min exposure to the monolayers. As shown in Figure 2B, in EP_1-HEK cell monolayers, the medium PGE_2 concentrations at 10 min were not different from the initial concentrations, indicating that, in the absence of PGT, the added PGE_2 remained in the extracellular compartment. Treatment with T26A (5 μmol/L) did not affect medium PGE_2 levels in EP_1-HEK cells. However, in PGT-EP_1-HEK cells, the medium PGE_2 concentration after 10 min exposure to the monolayer was significantly reduced compared to that of control cells and, in these cells, acute inhibition of PGT with T26A returned medium PGE_2 levels at 10 min to levels similar to those in EP_1-HEK cells (Fig. 2B). Taken together, the data of Figure 2 indicate that PGT expression at the cell membrane affects both PGE_2 availability for signaling at the cell surface as well as the bulk-fluid phase PGE_2 concentration.

PGT controls endogenous PGE_2 bioavailability and modulates autocrine/paracrine PGE_2 signaling via the EP_1 receptor

Cells synthesize and release endogenous PGE_2 in response to diverse stimuli, such as bradykinin (Miller 2006). We have shown previously that PGT modulates the net release of PGE_2 into the bulk cell culture medium in MDCK cells in response to bradykinin (Nomura et al. 2005). Here, we confirmed these results in HEK cells. We treated EP_1-HEK and PGT-EP_1-HEK cells with bradykinin (10 μmol/L) in the absence and presence of the PGT blocker T26A (5 μmol/L). As shown in Figure 3A, although bradykinin-induced PGE_2 release from both EP_1-HEK and PGT-EP_1-HEK cells, the PGE_2 concentration in the medium bathing PGT-EP_1-HEK cells was

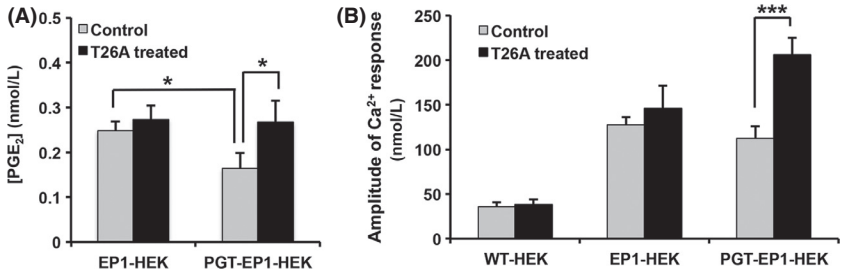

Figure 3. PGT modulates EP_1 Ca^{2+} responses induced by autocrine/paracrine PGE_2 signaling. (A) PGE_2 concentrations in media of EP_1-HEK and PGT-EP_1-HEK cells treated with 10 μmol/L bradykinin in the absence or presence of 5 μmol/L T26A for 30 min at 37°C. Medium [PGE_2] was lower in cells expressing PGT, but not when PGT was inhibited with T26A. Values are mean ± SEM ($n = 3$ for each experiment). *$P < 0.05$. (B) Bradykinin-induced EP_1 signaling (Ca^{2+} responses) in WT-HEK, EP_1-HEK, and PGT-EP_1-HEK cells. EP_1-expressing cells had a Ca^{2+} response to bradykinin above that of wild-type cells. In cells that were also expressing PGT, the PGT inhibitor T26A doubled the degree of signaling via EP_1. Data are mean ± SEM ($n \geq 4$ for each experiment), with >100 cells analyzed per experiment and per experimental condition. *$P < 0.05$, ***$P < 0.001$.

significantly lower than that in the medium bathing EP_1-HEK cells. Inhibiting PGT with T26A blocked the effect of PGT to lower PGE_2 in the medium (Fig. 3A). These results thus confirm those we reported previously using MDCK monolayers (Nomura et al. 2005).

Next, we examined the ramifications of this PGT effect on autocrine EP_1 signaling. Bradykinin induces intracellular Ca^{2+} release in HEK 293 cells by activating B2 receptors (Kramarenko et al. 2009). All three cell lines (WT-HEK, EP_1-HEK, and PGT-EP_1-HEK) responded to bradykinin with an increase in intracellular Ca^{2+} levels, although the amplitude of the response was about threefold higher ($P < 0.05$) in the two cell lines overexpressing the EP_1 receptor compared to wild-type cells (Fig. 3B). Thus, most of the bradykinin-induced Ca^{2+} response in EP_1-HEK and PGT-EP_1-HEK cells results from signaling through EP_1. The amplitude of the Ca^{2+} response to bradykinin in PGT-EP_1-HEK cells doubled when these cells were treated with the PGT blocker T26A (Fig. 3B). Taken together, the data of Figure 3 indicate that PGT controls the concentration of extracellular PGE_2 in this autocrine signaling system, and that the resulting cell surface PGE_2 concentration is reflected in the degree of signaling through EP_1 receptors.

PGT modulates autocrine/paracrine signaling through the EP_4 receptor

In addition to Ca^{2+}, another important second messenger for PGE_2 is intracellular cAMP, resulting from activation of either the EP_2 or EP_4 receptor by PGE_2. We tested the hypothesis that, as with the EP_1 receptor, PGT regulates EP_4-mediated autocrine/paracrine signaling. We constructed a cell system consisting of wild-type MDCK cells ("WT-MDCK") or MDCK cells that were stably transfected with PGT tagged with GFP (green fluorescent protein) at the carboxyl terminus ("PGT-GFP-MDCK") (Endo et al. 2002). These cell lines were then transiently transfected with an EP_4 receptor-expressing cDNA. As above, we stimulated both cell lines with bradykinin so as to induce acute, autocrine/paracrine signaling of EP_4 receptors by endogenous PGE_2.

As shown in Figure 4A, although bradykinin-induced the net release of PGE_2 from both cell lines, expression of PGT significantly reduced the extracellular PGE_2 concentration compared to control, whereas blocking PGT activity with T26A (5 μmol/L) reversed this reduction. As shown in Figure 4B, bradykinin-induced intracellular cAMP production was significantly lower in PGT-GFP-MDCK cells compared to controls, and T26A treatment reversed this effect. These findings demonstrate that, as with EP_1 signaling via intracellular Ca^{2+}, PGT regulates autocrine/paracrine signaling by the EP_4 receptor via cAMP.

PGT modulates homologous desensitization of the EP_4 receptor in response to exogenous PGE_2

The EP_2, EP_3, and EP_4 receptors undergo homologous desensitization in response to pharmacological addition of extracellular PGE_2 (Negishi et al. 1993; Nishigaki et al. 1996; Ashby 1998). To test the hypothesis that PGT modulates agonist-induced EP_4 desensitization, we transiently transfected WT-MDCK cells and PGT-GFP-MDCK cells with the human EP_4 receptor cDNA and determined cell surface EP_4 expression by a ligand-binding assay in response to exogenous PGE_2 (0–50 nmol/L).

As shown in Figure 5A, in WT-MDCK cells ("WT"), exogenous PGE_2 caused dose-dependent EP_4 desensitization, such that exposure to 50 nmol/L PGE_2 induced desensitization of about 50% of cell surface EP_4 receptors.

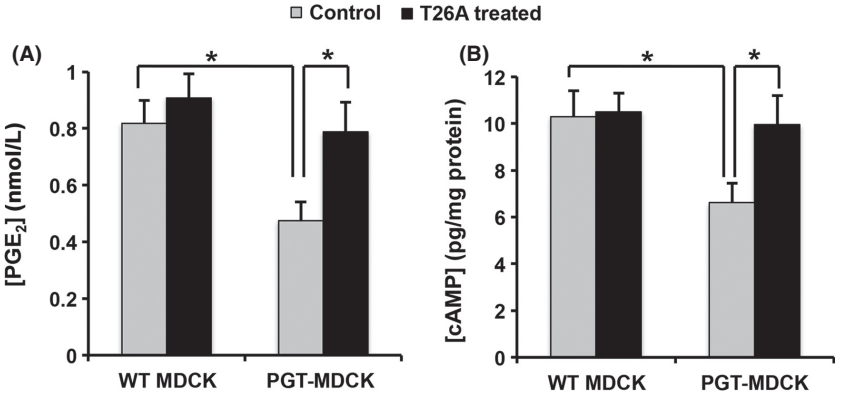

Figure 4. PGT regulates cAMP accumulation in response to autocrine/paracrine EP_4-mediated PGE_2 signaling. WT-MDCK and PGT-GFP-MDCK cells were transiently transfected with the EP_4 receptor cDNA for 24 h, then incubated for 18 h with 250 μmol/L IBMX, and afterward treated with 10 μmol/L bradykinin in the absence or presence of 5 μmol/L T26A for 30 min. (A) PGE_2 concentrations in the media. (B) Intracellular cAMP accumulation. PGT expression reduced both the concentration of PGE_2 in the medium and the accumulation of intracellular cAMP in response to 30 min exposure to bradykinin. Both of these PGT effects were reversed by the PGT inhibitor T26A. Values are mean ± SEM (n = 3 for each experiment). *P < 0.05.

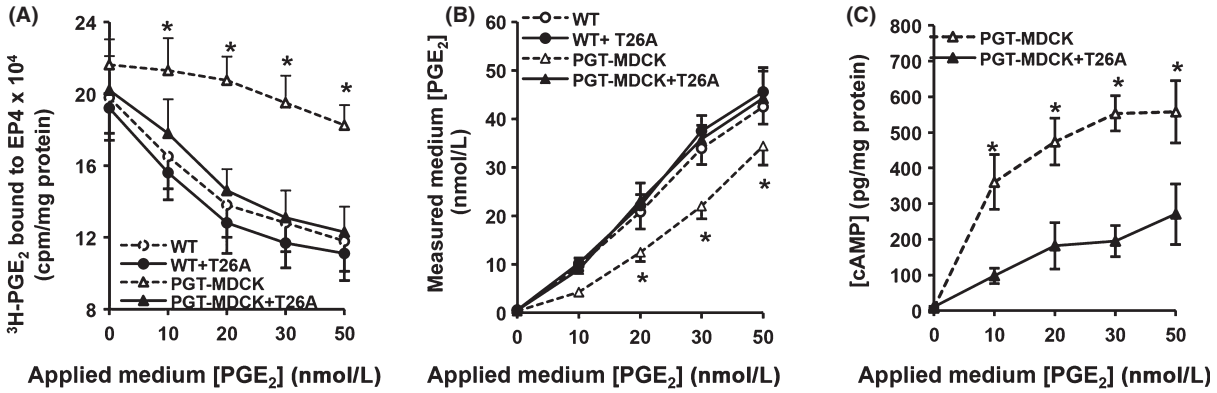

Figure 5. PGT regulates EP_4 receptor desensitization in response to exogenous PGE_2. Wild-type MDCK ("WT") and PGT-GFP-MDCK ("PGT-MDCK") cells were transiently transfected with the EP_4-expressing cDNA, and 24 h later were exposed to pharmacological concentrations of exogenous PGE_2 (0–50 nmol/L, "applied medium [PGE_2]") in the presence or absence of 5 μmol/L T26A. After 10 min, media were collected for determining residual extracellular PGE_2 concentrations by ELISA, and cells were harvested for quantifying the amount of cell surface EP_4 receptor using a binding assay. (A) Cell surface EP_4 receptor. Exogenous PGE_2 caused a reduction in cell surface EP_4 (i.e., desensitization) in wild-type MDCK cells (open circles); T26A had no effect on this desensitization (closed circles). When PGT was expressed, it abrogated the ability of PGE_2 to cause EP_4 desensitization (open triangles), an effect that was reversed by the PGT inhibitor T26A (closed triangles). (B) Media [PGE_2] at the conclusion of 10 min exposure to cell monolayers. PGT reduced the concentration of PGE_2 in the media (open triangles) compared to the lack of change in wild-type cells (open circles) or wild- type cells exposed to T26A (closed circles). T26A reversed the ability of PGT to lower media [PGE_2] (closed triangles). (C) Intracellular cAMP accumulation. PGT-GFP-MDCK cells ("PGT-MDCK") were transfected with EP_4 as above, treated with 250 μmol/L IBMX for 18 h, and exposed to exogenous PGE_2 ± T26A for 10 min at various doses as above. Cells expressing uninhibited PGT (absent T26A, open triangles) exhibited a robust cAMP dose-response to PGE_2, whereas PGT-MDCK cells in which PGT was inhibited by T26A (open triangles) exhibited a significantly blunted cAMP response to exogenous PGE_2. Values are mean ± SEM (n = 3 for each experiment). *P < 0.05.

In contrast, in cells expressing PGT ("PGT-MDCK"), PGE_2-induced EP_4 desensitization was significantly abrogated. Although, T26A (5 μmol/L) had no effect on PGE_2-induced EP_4 desensitization in WT-MDCK cells, it inhibited PGT-mediated EP_4 retention on the cell surface.

PGT-mediated modulation of EP_4 desensitization involves PGT-mediated removal of exogenous PGE_2, as shown in Figure 5B. The measured PGE_2 concentration in the medium 10 min after PGE_2 addition to the cell monolayer was significantly lower in PGT-GFP-MDCK cells compared to that in WT-MDCK cells. When PGT

was acutely inhibited by T26A, extracellular PGE_2 levels measured in media exposed to PGT-GFP-MDCK cells were not different from those observed using WT-MDCK cells (Fig. 5B).

The downstream signaling consequences of these changes in EP_4 receptor desensitization are shown in Figure 5C. In PGT-GFP-MDCK cells, PGE_2 increased intracellular cAMP accumulation in a dose-dependent manner to a degree that was significantly greater when PGT was active than when it was inhibited (Fig. 5C).

Together, the data presented in Figure 5 suggest a model in which plasma membrane PGT determines the cell surface PGE_2 concentration which, in turn, determines the degree of short-term, agonist-induced desensitization. The degree of desensitization, in turn, determines the degree of intracellular cAMP accumulation in response to exogenous PGE_2.

Discussion

The present studies show that the PGT modulates PGE_2 signaling by altering ligand concentration at cell surface receptors. Our findings provide a molecular mechanism for several recent reports in which acute or chronic manipulation of PGT transporter activity altered downstream physiological events. These include relaxation of tracheal smooth muscle by stimulants of protease-activated receptor-2 (Henry et al. 2005), astrocyte-mediated cerebral vasodilation (Gordon et al. 2008), regulation of preadipocyte aromatase activity by PGE_2 (Subbaramaiah et al. 2011), and maintenance of a functional corpus luteum (Lee et al. 2013).

Our results may also provide insight into the mechanisms underlying failure of postnatal closure of the ductus arteriosus in mice (Chang et al. 2010), and of pachydermoperiostosis in humans, both of which result from homozygous null PGT alleles (Busch et al. 2012; Diggle et al. 2012; Sasaki et al. 2012; Seifert et al. 2012; Zhang et al. 2012). Although, as described above, an argument can be made that these phenotypes represent PGE_2 acting systemically as a hormone, the present results suggest that at least some of the vascular, skeletal, and/or dermal phenotypes resulting from PGT inactivation may represent abnormal autocrine/paracrine PGE_2 signaling.

The ability of the PGT transporter to compete with EP receptors for ligand is dependent on the respective abilities of the transporter and the receptors to bind, and turn over, the ligand. The binding constant of PGE_2 to PGT is about 90 nmol/L (Kanai et al. 1995), and that of PGE_2 for EP_1 and EP_4 is in the range of 2–20 nmol/L (Narumiya et al. 1999). Although PGT binds PGE_2 more weakly than does EP_1 or EP_4, it is apparently able to compete for the ligand even when EP receptors are overexpressed, suggesting that PGT likely turns over PGE_2 faster than do the receptors.

EP_1 signaling is mediated by intracellular Ca^{2+} release. Regardless of whether the source of PGE_2 was endogenous (i.e., postbradykinin) or exogenous, PGT lowered the amount of cell surface PGE_2 and reduced EP_1 signaling (Figs. 2, 3). Similarly, PGT reduced EP_4-mediated intracellular cAMP accumulation when the source of PGE_2 was endogenous (postbradykinin) (Fig. 4). However, when exogenous PGE_2 in pharmacological concentrations was introduced, EP_4 exhibited rapid desensitization in accord with prior studies (Nishigaki et al. 1996). In that case, PGT reduced cell surface PGE_2 and abrogated EP_4 desensitization, resulting in an increase in cAMP formation (Fig. 5). Thus, in the case of EP_4 signaling, the net result of competition for ligand between PGT and the EP_4 receptor depends on the source, timing, and concentration of the cell surface PGE_2.

As above, we have previously drawn an analogy between neurotransmitter release/reuptake at the synapse and PG release/reuptake by cells engaged in autocrine/paracrine PG signaling (Nomura et al. 2005). The present studies allow us to extend this analogy insofar as we now show that PGT, like neurotransmitter reuptake carriers, regulates availability of ligand for its GPCR. The analogy is of further interest in that both neurotransmitter and PG reuptake carriers are regulated by their respective ligands. Thus, dopamine activates the dopamine reuptake carrier through dopamine receptors (Zapata et al. 2007), and the PGT substrate $PGF_{2\alpha}$ inhibits PGT through the FP receptor (Vezza et al. 2001).

In the same way that inhibitors of neurotransmitter reuptake have been useful in the management of psychiatric diseases (Mann 2005), inhibitors of prostaglandin reuptake, by raising endogenous PG levels (Chi et al. 2011; Syeda et al. 2012), might be medicinally applicable for conditions in which exogenous prostaglandins are currently used, such as glaucoma (Stjernschantz 2004), pulmonary artery hypertension (Gomberg-Maitland and Olschewski 2008), and vascular insufficiency (Amendt 2005). Because we found that the PGT inhibitor T26A increased autocrine/paracrine signaling through EP_1 and EP_4 receptors only in the presence of PGT, it appears that T26A does not interact directly with these two PGE_2 receptors. Thus, T26A or its derivatives may offer a small-molecule approach to raising endogenous prostaglandin levels.

Finally, we note that there are currently approximately 120 orphan GPCRs (Civelli 2005; Oh et al. 2006) and 90 orphan SLC transporters (Dahlin et al. 2009; Schlessinger et al. 2010), all with unassigned ligands. The present findings show that regulatory competition between GPCRs and SLC carriers occurs outside of the synaptic cleft, and

thus may represent a more general phenomenon than previously appreciated.

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Disclosures

None declared.

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