Extracellular Loop II Modulates GTP Sensitivity of the Prostaglandin EP3 Receptor

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ABSTRACT

Unlike the majority of G protein–coupled receptors, the prostaglandin E2 (PGE2) E-prostanoid 3 (EP3) receptor binds agonist with high affinity that is insensitive to the presence of guanosine 5′-(3-thio)triphosphate (GTPγS). We report the identification of mutations that confer GTPγS sensitivity to agonist binding. Seven point mutations were introduced into the conserved motif in the second extracellular loop (ECII) of EP3, resulting in acquisition of GTP–sensitive agonist binding. One receptor mutation W203A was studied in detail. Loss of agonist binding was observed on intact human embryonic kidney 293 cells expressing the W203A receptor, conditions where high GTP levels are present; however, high affinity binding [3H]PGE2 was observed in broken cell preparations washed free of GTP. The [3H]PGE2 binding of W203A in broken cell membrane fractions was inhibited by addition of GTPγS (IC50 21 ± 1.8 nM). Taken together, these results suggest that the wild-type EP3 receptor displays unusual characteristics of the complex coupled equilibria between agonist-receptor and receptor–G protein interaction. Moreover, mutation of ECII can alter this coupled equilibrium from GTP-insensitive agonist binding to more conventional GTP-sensitive binding. This suggests that for the mutant receptors, ECII plays a critical role in linking the agonist bound receptor conformation to the G protein nucleotide bound state.

Introduction

The arachidonic acid metabolite prostaglandin E2 (PGE2) mediates diverse physiologic responses via its interactions with specific seven transmembrane G protein-coupled receptors (GPCRs). Four distinct PGE2 receptor subtypes, upon the basis of ligand selectivity and signal transduction pathways activated, have been classified as E-prostanoid 1 (EP1), EP2, EP3, and EP4 (Coleman et al., 1994; Hata and Breyer, 2004). Physiologic evidence suggests that the rabbit EP3 receptor signals via inhibition of cyclic AMP (cAMP) generation in vivo (Sonnenburg et al., 1990), and the cloned rabbit, mouse, bovine, and human receptors were shown to signal through this pathway when expressed in cell culture systems (Sugimoto et al., 1992; Namba et al., 1993; Audoly and Breyer, 1997a). Additional alternate EP3-evoked signal transduction pathways have been described (Sugimoto et al., 1992, 1993; Irie et al., 1993; Namba et al., 1993; Negishi et al., 1993a,b), including a pertussis toxin-insensitive pathway that increased cAMP response elements (CRE) reporter activity (Audoly et al., 1999).

Ligand binding to GPCRs is a coupled equilibrium dependent on both the ligand, agonist, antagonist, or inverse agonist as well as the interaction of the receptor with G proteins. The classic ternary complex model describes the interconversion of the inactive conformation of the receptor R to the active conformation of the receptor R* facilitated by the presence of G proteins and modulated by the presence of GTP or GTP analogs (De Lean et al., 1980; Samama et al., 1993). Agonist binds with high affinity to the active R* state, and a concomitant exchange of guanosine diphosphate (GDP) for GTP on the associated G protein initiates the intracellular signaling cascade. In the ternary complex model, addition of nonhydrolyzable GTP analogs such as guanosine 5′-(3-thio)triphosphate (GTPγS) causes an affinity shift of agonist from a high-affinity state to a low-affinity state. Classic studies by De Lean et al. (1980) showed that the β-adrenergic receptor has both high- and low-affinity sites differing in affinity by 100-fold. Addition of GTP analogs dramatically shifted the population of receptors to the low-affinity state for the β-adrenergic receptor, leading to a loss of radiolabeled agonist binding (Williams and Lefkowitz, 1977). While this model explains the behavior of some classes of

ABBREVIATIONS: BSA, bovine serum albumin; cAMP, cyclic AMP; [cAMP], intracellular cyclic AMP; CRE, cAMP response elements; DMEM, Dulbecco’s modified Eagle’s medium; EC, extracellular loop; Endo H, endoglycosidase H; EP, E-prostanoid; ER, endoplasmic reticulum; FBS, fetal bovine serum; GDP, guanosine diphosphate; GPCR, G protein–coupled receptor; GTPγS, guanosine 5′-[gamma]-O-(3-thio)triphosphate; HA, hemagglutinin; HEK, human embryonic kidney; HRP, horseradish peroxidase; PE, phycoerythrin; PGE2, prostaglandin E2; PNGase F, N-glycosidase F; TBS, Tris-buffered saline; TBST, Tris-buffered saline/Tween 20; WT, wild-type.
GPCRs, the behavior of other GPCRs does not easily fit this model. Several GPCRs have been shown to have little or no affinity shift upon the addition of nonhydrolyzable GTP analogs, including the histamine H3 receptor, the PACAP receptor, and the melatonin Mel1A receptor (Roka et al., 1999; Hann et al., 2004; Muller et al., 2007). For these receptors, agonist binding is insensitive to the presence of nonhydrolyzable GTP analogs.

The agonist affinity for recombinant mouse prostaglandin EP3 receptor was reported to be relatively insensitive to the presence of GTPγS binding and found to increase or decrease the affinity for PGE2, depending on the splice variant tested (Sugimoto et al., 1993). The changes in affinity were quite modest, resulting in only a 2- or 3-fold change in agonist affinity. A central question is whether the EP3 receptor displays true high- and low-affinity binding, whether the high-affinity agonist binding is dependent on the presence of G proteins, and what the consequences of dissociation of the G protein are on agonist binding.

To address these questions, we compared the agonist binding and signaling properties of wild-type (WT) and mutant EP3 receptors. Analysis of the amino acid sequences of the cloned prostaglandin receptors has identified regions of characteristic, conserved amino acid sequences, including a sequence of eight amino acid residues clustered in the amino-terminal portion of the second extracellular loop (ECII), including an invariant triplet Trp-Cys-Phe (Pierce et al., 1995; Audoly and Breyer, 1997a). Mutation of ECII of the EP3 receptor leads to gain-of-function of C1 methyl ester ligands and may affect receptor-ligand interactions either directly or indirectly. Here, we demonstrate that mutation of conserved residues in ECII alters GTP sensitivity and agonist-evoked signal transduction. These results suggest that the conformation of ECII is critical in sensing the nucleotide bound state of the G protein.

Materials and Methods

Expression of the EP3 Receptor in Cell Culture. Mutation of the hemagglutinin (HA)-tagged EP3 receptor and plasmid generation was previously described elsewhere (Audoly and Breyer, 1997a). The mutant nomenclature has been updated from the original report to agree with current standards; for example, WA203 is now designated W203A. Human embryonic kidney (HEK)293 cells (5 × 10^6 cells) plated at approximately 50% confluence were transfected with 3 μg of the receptor cDNA of interest and 3 μg of pCRIIE2acZ plasmid (a kind gift from Dr. R. Cone, Vanderbilt University) (Chen et al., 1995) using lipofectamine-2000 (Invitrogen, Carlsbad, CA). Six hours after the addition of DNA-Lipofectamine complex, the medium was aspirated and replaced with Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen), containing 10% fetal bovine serum (FBS). For reporter assays, 24 hours after transfection cells were plated in 96-well plates at a density of 5 × 10^3 cells/well in 100-μl DMEM/10% FBS/20 μM indomethacin containing 5 mM sodium butyrate, and incubated an additional 12 to 16 hours, at which point the cells had reached confluence. In some cases, polyclonal cell lines were selected from cells transiently transfected with plasmids bearing WT or mutant EP3 cDNAs. Briefly, transfected cells were cultured for 3 to 4 days in DMEM medium with 10% FBS, and upon reaching confluence, 600 μg/ml G418 antibiotic was added for selection. The G418 level was gradually reduced to 500 μg/ml over several days, and then cell lines were maintained in medium containing 400 μg/ml G418 thereafter. Monoclonal lines were isolated from polyclonal pools by limiting dilution.

Flow Cytometry Analysis. Cell surface expression levels of receptors in the 77A WT and W203A mutants were analyzed by flow cytometry using a FACSArria Cell Sorting system (BD Biosciences, San Jose, CA). The EP3 receptor fused with HA-tag on the extreme N terminus of the receptor was detected by using monoclonal anti-HA antibody (Cell Signaling Technology, Beverly, MA) with phycoerythrin (PE) conjugated anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) and sorted by fluorescence intensity. Cells incubated with secondary antibody alone served as controls.

Cell Surface ELISA. HEK293 monoclonal 77A #47 WT (clone 47), W203A #102 (clone 102), and vector #5 (clone 5) cells were plated into poly-L-lysine coated 24-well plate (200,000 cells/well) in DMEM complete medium containing 5 mM sodium butyrate and 20 μM indomethacin and were cultured for 18 to 24 hours in CO2 incubator at 37°C. All subsequent incubations were performed at 23°C. Cells were then washed three times with Tris-buffered saline (TBS), and wells were blocked with 1% bovine serum albumin (BSA)/TBS for 30 minutes and then incubated with mouse monoclonal anti-HA primary antibody (1:1000) (Cell Signaling Technology) for 1 hour. Cells were washed three times with TBS and were again blocked with 1% BSA/TBS for 15 minutes. Cells were incubated with horseradish peroxidase (HRP) conjugated anti-mouse secondary antibody (1:1000) (Jackson Immunoresearch Laboratories) in 1% BSA/TBS for 1 hour, and washed 3 times with TBS; the plate was then developed with hydrogen peroxide and 2,2’-azino-bis-[3-ethylbenzolene-6-sulfonic acid] (ABTS; Bio-Rad Laboratories, Hercules, CA). The reaction was terminated by addition of 2% oxalic acid, and the absorbance was measured at 415 nm.

Confocal Immunocytochemistry. Monoclonal WT, W203A mutant and vector cells were plated into glass-bottom chamber slides in DMEM medium containing 5 mM sodium butyrate and 20 μM indomethacin, and they were cultured 16 hours at 37°C in the CO2 incubator. Cells were washed with PBS, fixed with 4% paraformaldehyde at room temperature for 10 minutes, and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, Saint Louis, MO) for 5 minutes at room temperature. Wells were washed with PBS, blocked with 5% normal goat serum and 1% BSA in PBS for 40–60 minutes at 23°C, and then washed twice with TBS. Double staining was performed using antibodies specific for either endoplasmic reticulum (ER) (Abcam, Cambridge, MA), antirecycling endosomes (Lapierre et al., 2007), or anti-Golgi (Abcam) and colocalized with anti-HA (1:100 dilution) antibodies in 1% BSA in TBS and incubated 16 hours at 4°C. Wells were washed three times for 5 minutes each with TBS. Fluorescently labeled Alexa-488 (for ER, endosome and Golgi) and Alexa-568 (for HA) secondary antibodies (1:500) (Invitrogen) were incubated in 1% BSA/TBS for 30 to 45 minutes at room temperature in the dark. Wells were washed 3 times with TBS and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) in low lighting. Images were obtained using a Zeiss Inverted LSM510 microscope (Carl Zeiss Microscopy, Thornwood, NY).

Cell Surface Biotinylation. Cell surface biotinylation was performed according to the manufacturer's instructions (Pierce cell-surface protein isolation kit; Thermo Fisher Scientific, Rockford, IL). An equal number of monoclonal WT, W203A, and vector cells were plated in T75 cell culture flasks in DMEM medium containing 5 mM sodium butyrate and 20 μM indomethacin; they were cultured overnight at 37°C in the CO2 incubator. Cell surface biotinylation was performed using Sulfo-NHS-SS-Biotin for 15 minutes at 4°C, and the reaction was stopped by the addition of quenching solution supplied by the company. Collected cells were solubilized with lysis buffer and the lysate was centrifuged to eliminate the insoluble fraction. The supernatant with biotinylated proteins was incubated with immobilized NeutraAdvin gel slurry for 60 minutes at room temperature, and the beads were then extensively washed with wash buffer containing protease inhibitors. Proteins were eluted with SDS-PAGE sample buffer containing 50 mM dithiothreitol for 60 minutes at room temperature and used the eluted sample for Western blot analysis.
Western Blot Analysis. Biotinylated protein samples collected from monolonal WT, W203A and vector cells were analyzed for cell-surface expression of glycosylated EP3 receptors. In some cases, the biotinylated WT and mutant samples were treated with endoglycosidase H (Endo H), which cleaves the chitobiase core of high mannose and hybrid oligosaccharides from N-linked glycoproteins or with N-glycosidase F (PNGase F), which cleaves between the innermost GlcNAc and asparagine residues of high mannosid, hybrid, and complex oligosaccharides from N-linked glycoproteins. Equal amounts of biotinylated or enzyme treated samples were loaded into 10% SDS-PAGE. Resolved proteins were transferred onto a nitrocellulose membrane. The filter was then incubated overnight with anti-HA mouse monoclonal (1:200) primary antibody at 4°C. The blot was washed with ice-cold Tris-buffered saline/Tween 20 (TBST), and incubated with HRP-conjugated anti-mouse secondary antibody (1:2000) for 60 minutes at room temperature and then develop with ECL reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA).

To validate the cell-surface selectivity of the biotinylation, Western blot analysis was performed in the biotinylated samples of Rho-GDI, one of the predominantly expressed cytosolic proteins. Lysate collected from untreated (nonbiotinylated) cells served as a negative control. Samples were resolved in 10% SDS-PAGE, and the proteins were transferred onto a nitrocellulose membrane. The filter was incubated with anti-Rho-GDI rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody (1:250) overnight at 4°C. Similarly, control and urea treated samples were resolved in 10% SDS-PAGE to detect Goα protein using a 1:2500 dilution of rabbit anti-Goα antibody (Carlson et al., 1989) and incubated overnight with 3% milk in TBST at 4°C. Filters were then washed with ice-cold TBST, and incubated with HRP conjugated anti-rabbit secondary antibody (1:20000) (Jackson ImmunoResearch Laboratories) for 60 minutes at room temperature. The blots were developed with ECL reagent.

Radioligand Binding on Intact Cells. Monolonal 77A WT, W203A, and vector transfected HEK293 cells were plated into a poly-L-lysine coated 24-well plate in complete DMEM medium containing 5 mM sodium butyrate and 20 μM indomethacin and were cultured for 18 to 24 hours in a CO2 incubator at 37°C. Cells were washed once with ice-cold PBS and incubated with 200 μl of 2 nM [3H]PGE2 (PerkinElmer Life and Analytical Sciences) in the presence or absence of (2)-7-[(1R,2R,3R)-3-hydroxy-2-[1R,2R,3R]-3-hydroxy-4-(phenoxy)but-1-eny]-5-oxocyclopentyl]-N-methyl sulfonl hept-5-enamide (sulprostone; 5 μM) for 2 hours at 4°C. Similarly, control and urea treated samples were resolved in 10% SDS-PAGE to detect Goα protein using a 1:2500 dilution of rabbit anti-Goα antibody (Carlson et al., 1989) and incubated overnight with 3% milk in TBST at 4°C. Filters were then washed with ice-cold TBST, and incubated with HRP conjugated anti-rabbit secondary antibody (1:20000) (Jackson ImmunoResearch Laboratories) for 60 minutes at room temperature. The blots were developed with ECL reagent.

Radioligand Binding on Isolated Membranes. Membranes from stable HEK293 cell lines transfected with 77A WT, W203A, or empty vector were prepared after incubation of the cells with 5 mM sodium butyrate for 24 hours. After hypotonic lysis, the lysate was layered on a 60% sucrose cushion and centrifuged at 150,000 g for 1 hour at 4°C. The membrane fraction collected and the protein concentration were determined by the BCA assay (Thermo Fisher Scientific). For saturation-binding isotherms, equal volumes of control and urea-treated membranes collected from WT and W203A were analyzed for one point [3H]PGE2 binding in the presence or absence of 5 μM sulprostone. In some studies, urea-washed membranes were preincubated with GDP (10 μM) and heterotrimeric G proteins (Goαi, 10 μg; Gβγ, 10 μg) and tested for ligand binding in the presence or absence of GDP or PGE2 (10 μM).

CRE Reporter Assay. Transfected cells were plated in DMEM/10% FBS/20 μM indomethacin (2-[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid) containing 5 mM sodium butyrate in 96-well plates at 5 x 10^4 cells per well. Sulprostone dissolved at varying concentrations in OPTI-MEM containing 20 μM indomethacin were added to cells and incubated for an additional 6 hours at 37°C in 5% CO2. Medium was aspirated and β-activity was measured as described elsewhere (Sheffer and Conn, 2008). Monolonal HEK293 expressing 77A WT, W203A, or empty vector (60,000 cells/well) were plated into poly-L-lysine coated 24-well plate in complete DMEM medium containing 5 mM sodium butyrate and 20 μM indomethacin and were cultured for 16 to 18 hours in CO2 incubator at 37°C. The media were replaced with serum-free DMEM containing 20 mM HEPES and incubated at 37°C for 2 hours, pretreated with 0.5 mM IBMX in stimulation buffer (DMEM containing 15 mM HEPES, 0.025% ascorbic acid) at 37°C for another 30 minutes, and then stimulated with either vehicle or 1 μM isoproterenol for another 20 minutes. The reaction was terminated by the addition of ice-cold 3% trichloroacetic acid (TCA) and incubated at least 2 hours at 4°C. Competition binding was performed incubating 15-μl TCA extract, 35 μl of 15.71 nM [3H]cAMP, and 500 μl of assay buffer (100 mM Tris-HCl, pH 7.4; 100 mM NaCl and 5 mM EDTA) containing 0.2 μg/ml cAMP-binding protein isolated from bovine adrenal cortices. The binding reaction was incubated on ice for 2 hours, and the reaction was terminated by vacuum filtration over GF/F filters and counted in a liquid scintillation counter.

Data Analysis. All data are presented as the mean ± S.E.M. of at least three independent experiments. Statistical analyses were performed using the Student’s t test. A two-tailed value of P < 0.05 was considered statistically significant. KD and Bmax values for saturation isotherm radioligand-binding experiments were calculated based on a one-site binding model using Prism software (GraphPad Software, Inc., San Diego, CA). EC50 values for CRE signal transduction experiments were calculated based on a sigmoidal dose-response model using Prism software (GraphPad Software, Inc., San Diego, CA).

Results

Agonist Affinity to the Rabbit EP3 Receptor Is Insensitive to GTP Analogos. The rabbit receptor has not been characterized with respect to GTP sensitivity of agonist binding. As seen in Fig. 1, saturation isotherm binding experiments on total membrane fractions revealed single-site high-affinity specific binding of [3H]PGE2. Addition of GTPγS did not result in a statistically significant change in...
Ligand-binding affinity ($K_D$ WT $= 1.6 \pm 0.3$ nM, $K_D$ WT, + GTPγS $= 2.6 \pm 0.6$ nM, N.S.) (Fig. 1). Thus, similar to findings reported for the mouse EP3 receptors, agonist binding to the rabbit EP3 receptor is insensitive to the presence of GTP analogs.

**Loss of Signal Transduction upon Mutation of ECII.**

The ability of the WT rabbit EP3 receptor 77A splice variant to evoke an agonist-dependent increase in CRE reporter activity was compared with that of receptors bearing a series of point mutations in the conserved sequence in the ECII (Fig. 2, A and B). HEK293 cells transiently transfected with WT EP3 receptor and stimulated with the EP3 agonist sulprostone increased CRE reporter activity in a dose-dependent manner, with an EC$_{50}$ of 12.1 ± 3.8 nM, consistent with previous published findings (Audoly et al., 1999). Each point mutation tested caused a reduction in the EC$_{50}$ for reporter activation. In some cases, the reduction was modest, such as the receptors bearing the point mutations Q198A (EC$_{50}$ $= 68.8 \pm 39$ nM; $P < 0.0001$), W199A (EC$_{50}$ $= 93.6 \pm 46$ nM; $P = 0.0008$), or more dramatic as in T202A (EC$_{50}$ $= 2.2 \pm 1.1$ µM; $P < 0.0001$). In the remaining mutations, the cells transfected with EP3 receptors bearing P200S, W203A, C204A, and F205A substitutions, the response to sulprostone in the CRE reporter assay was minimal or absent at agonist concentrations up to 30 µM. This is despite the fact that each of these receptors, with the exception of P200S, has been previously demonstrated to bind the agonist sulprostone with affinity equal to or greater than that observed for the WT receptor in

![Fig. 1](image1.png)

**Fig. 1.** The effect of GTPγS on agonist affinity of the rabbit EP3 77A receptor. Saturation isotherm analysis was performed in monoclonal cell membranes of the WT EP3 receptor in the absence (○) or presence (□) of 100 µM GTPγS. Data presented are from a single experiment performed in duplicate, and are representative of three independent experiments.

![Fig. 2](image2.png)

**Fig. 2.** Mutation and signal transduction of ECII of the rabbit EP3 77A receptor. (A) A stretch of amino acids in the ECII is highly conserved among prostaglandin receptors. Selected residues were individually mutated to alanine as indicated, except for the proline at position 200, which was mutated to serine, the residue found in the FP receptor. (B) Analysis of CRE reporter signal transduction was performed in cells expressing EP3 WT or receptors with mutation at seven other positions in the ECII. Left panel: HEK293 cells were transiently transfected with EP3 77A WT (■), Q198A (▲), W199A (▼), P200S (●), T202A (■), or pBluescript empty vector (○). Right panel: W203A (□), C204A (△), F205A (◇). In the right panel, the WT and vector curves are replotted from the left panel for comparison. (C) A whole-cell binding assay was performed in HEK293 cells transiently transfected with cDNA encoding the WT or mutant EP3 receptor. Intact cells were incubated with [3H]PGE2 in the presence or absence of 5 µM sulprostone, then washed, lysed with 1-N NaOH, and counted in scintillation fluid. The data shown in (B) are from a single experiment performed in triplicate and are representative of six to eight individual experiments. The data shown in (C) represent the mean ± S.E.M. of at least three independent experiments performed in duplicate.
In contrast, cells stably expressing W203A did not display binding site for \[^3H\]PGE2 (ing the EP3 WT receptor displayed a single high-affinity receptor cDNA clones. 

![Image](https://example.com/image.png)

**Fig. 3.** Receptor expression and signal transduction in monoclonal stable lines. (A) analysis of CRE-mediated signal transduction in monoclonal cell lines. Monoclonal cell lines WT (▲), mutant W203A (□), and vector (○) were stimulated with the indicated doses of sulprostone. (B) saturation isotherm analysis of intact cell-surface binding of WT monoclonal cells expressing WT (▲) or the W203A mutant EP3 receptor (□) incubated with \[^3H\]PGE2 in the presence or absence of 5 μM sulprostone. (C) saturation isotherm analysis performed in monoclonal cell membranes of W203A (□). Data presented in (A) are from a single experiment performed in triplicate and are representative of seven independent experiments. Data presented in (B) are combined from four independent experiments. Data presented in (C) are from a single experiment performed in duplicate and are representative of three independent experiments.
indistinguishable distribution of the EP3 WT and W203A mutant receptors in the sucrose gradient (Fig. 4B). Fractions containing plasma membrane or light vesicles were identified using antibodies to the EGF receptor on control cells with or without pretreatment with EGF to internalize the EGF receptor (Hertel et al., 1985) (Fig. 4C). Greater than 85% of the [3H]PGE2 radioligand binding was found in the dense membrane fraction of cells expressing either the EP3 WT or the W203A receptor proteins. This supports the notion that W203A receptor expressed at the cell surface is competent to bind PGE2, and is not rapidly internalized into light vesicles. Consistent with this finding, a cell-surface enzyme-linked immunosorbent assay (ELISA) using antibodies directed to the N-terminal HA tag on nonpermeabilized cells gave similar signals in this quantitative assay (Fig. 4D). We confirmed the cell-surface expression of the receptors on the monoclonal lines by confocal microscopy and evaluated both WT and mutant receptor transport to the plasma membrane using specific antibodies to colocalize ER, recycling endosomes, and Golgi compartments with EP3. The observed colocalization of ER, recycling endosome, and Golgi with EP3 in permeabilized cells suggests that both WT and mutant receptors were properly trafficked to the plasma membrane (Fig. 5, A, B, and C).

To test the posttranslational processing of the receptors at the plasma membrane, we performed cell-surface
biotinylation analysis for glycosylation. The extent of receptor glycosylation was assessed by treating the biotinylated samples with glycosidase Endo H and PNGase F, which cleave off the core or complex oligosaccharides from N-linked glycoproteins, respectively. Analysis of cell-surface biotinylated proteins confirmed that both WT and W203A cells expressed fully glycosylated receptors on the surface of the cells, and both WT and mutant receptors displayed similar patterns of glycosylation (Fig. 6). Taken together with antibody staining studies, these data provide compelling evidence that the correctly folded and processed receptor is found at the cell-surface plasma membrane for both WT and W203A receptors.

Agonist Binding to W203A Receptors Is GTPγS Sensitive. To test the hypothesis that the agonist binding of W203A was sensitive to the high intracellular GTP concentrations, [3H]PGE2 binding was performed on broken-cell membranes in the presence of increasing concentrations of GTPγS. No change in [3H]PGE2 binding was observed with concentrations of GTPγS up to 10 μM in WT, whereas the agonist binding to W203A was significantly inhibited by the addition of GTPγS (Fig. 7A). The GTPγS insensitivity of the WT receptor agonist-binding observed in these studies is consistent with results observed in the saturation-binding isotherm studies (Fig. 1). Agonist binding to W203A receptor was decreased with an EC50 value of 21 ± 1.8 nM, and complete inhibition of [3H]PGE2 binding was observed at 1 μM GTPγS. Because we had observed decreased cell-surface radioligand binding for each of the transiently expressed mutant receptors (Fig. 2C), the GTPγS sensitivity of agonist binding of these mutated receptors was determined. As shown in Fig. 7B, the receptors that displayed robust CRE signaling, WT and W199A, demonstrated GTPγS insensitive agonist binding, and Q198A had an intermediate reduction in agonist binding in the presence of GTPγS. In contrast, P200S, T202A, W203A, and F205A, which had reduced or absent signaling, each displayed profound reductions in agonist binding in the presence of GTPγS. Radioligand binding for receptor C204A was too low to assess the effect of GTPγS addition. The change in GTPγS sensitivity of the EP3 receptor is consistent with an altered interaction of the receptor with G protein. To test this hypothesis, G proteins were stripped from the membranes by washing with 4 M urea. As shown in Fig. 8A, urea-washed membranes prepared from cells expressing either WT and W203A receptors showed a loss of [3H]PGE2 binding. The loss of radioligand binding with urea washing suggests that the low-affinity R state that would be expected in the absence of G proteins is "nontrappable"; that is, it has a low affinity for PGE2 and a fast dissociation rate that is not detectable in filtration assays. Urea-washed membranes were reconstituted with purified heterotrimeric G protein, which partially restored radioligand binding. GTPγS agonist-binding sensitivity was observed in the reconstituted W203A-expressing membranes, while the membranes expressing the EP3 WT were insensitive to the addition of GTPγS. These data demonstrate that there is an intrinsic difference in the receptor G protein interaction and the response to GTPγS induced by mutations in the ECII region.

Receptors displaying GTP-insensitive agonist binding have been described as being "tightly coupled constitutively active"—that is, as having high agonist affinity binding due to the receptor being tightly associated with the G protein in the R* state, even in the absence of ligand. To test the constitutive activity of the receptor, cells expressing the EP3 receptor were transiently transfected with the Gs-coupled

![Fig. 5](image-url)
β2AR and stimulated with the β2AR agonist isoproterenol. Cells expressing the EP3 receptor had statistically significantly lower [cAMP]i levels when compared with vector-transfected stable cell lines, consistent with a tightly coupled constitutively active state for this receptor. Cells expressing the W203A receptor had [cAMP]i levels indistinguishable from WT, suggesting that although they have GTPγS-sensitive agonist binding there is no decrease in constitutive activity (Fig. 9).

Discussion

We describe here the interaction of the family A PGE2 EP3 receptor with G proteins and GTP. The rabbit EP3 receptor has at least five splice variants—72A, 74A, 77A, 80A, and NT—one of which can activate the CRE reporter with similar potency (Breyer et al., 1994; Audoly et al., 1999). These splice variants are identical except in the C-terminal intracellular sequence. The 77A splice variant of EP3 was studied in detail. The EP3 receptor has a high-affinity state with nanomolar affinity and a low-affinity state that is below the limit of detection of vacuum filtration. Mutation of ECII alters the coupled equilibrium of agonist binding and GTP-G protein interaction, making the receptor at least two orders of magnitude more sensitive to the presence of GTPγS. This suggests a key role for ECII in sensing the nucleotide bound state of the G protein in the cytoplasm.

Agonist affinity at the WT rabbit EP3 receptor is insensitive to the addition of GTPγS at concentrations up to 100 μM. This is consistent with observations made with endogenous EP3 receptors in rabbit kidney membranes (Sonnenburg et al., 1990). In contrast, physical dissociation of the G protein with urea leads to a loss of agonist binding, as has been previously described elsewhere for the adenosine receptor (May et al., 2005). The fact that PGE2 binding could be recovered, in part, by reconstitution with exogenously added purified G protein argues against urea-mediated disruption of the EP3 receptor structure per se, and suggests that the receptor-agonist affinity in the absence of G protein is below the level of detection.

Fig. 6. Glycosylation and cell-surface expression of WT and W203A. (A) Cell-surface biotinylated samples of WT, W203A, and vector clones were analyzed for receptor glycosylation. Western blot analysis indicates that comparable levels of fully glycosylated WT and mutant receptors are expressed at the cell surface. Treatment of samples with PNGase F cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. (B) Western blot analysis of Rho-GDI, an abundant cytosolic protein, as a control for cytosolic contamination. Data shown in (A) are from a single experiment and are representative of four independent experiments; data in (B) are representative of three independent experiments.

Mutations in the ECII region of the EP3 receptor were identified, which confer allosteric modulation of agonist binding by GTP and GTP analogs via interaction with the G protein. Addition of low concentrations of GTP analogs reduced the agonist affinity of the receptor below the level of detection using vacuum filtration. As a result, agonist binding of these mutant receptors was undetectable in intact cells where high intracellular concentrations of GTP are present; as a consequence, signal transduction was reduced or absent. These data suggest that the ECII region plays a key role in allosteric modulation of EP3 receptor function.

Fig. 7. GTPγS inhibits agonist binding in the W203A receptor. (A) Membranes prepared from stable HEK293 clones expressing WT ( ■) and W203A ( ■) were incubated with 4 nM [3H]PGE2 in the presence of varying concentrations of GTPγS. (B) One point binding analysis of membranes prepared from HEK293 cells transiently transfected with WT or mutant EP3 receptors. Specific binding of membranes was determined by incubation with [3H]PGE2 in the presence or absence of sulprostone (5 μM) (solid bars), and compared with the specific binding in the presence of 5 μM GTPγS (gray bars). Data are from a single experiment performed in duplicate and are representative of three or four independent experiments.
Activation of GPCRs requires alteration of the interhelical constraints that stabilize the inactive state to form a new set of contacts in the activated state. The free energy for this activation process comes from binding of the ligand, which in turn results in activation of the G protein (Kenakin, 1997; Gether and Kobilka, 1998; Lefkowitz, 2000; Akal-Strader et al., 2002). Although classic studies have focused on the role of the transmembrane helices in the binding of the agonist (Dohlman et al., 1987, 1988; Audoly and Breyer, 1997b), several studies have suggested a role for ECII in binding ligands. Studies reported here support a role for ECII in the interplay between ligand binding and the conversion of the receptor from the inactive (“R”) to the activated (“R*”) state. These studies suggest that, in addition to binding ligands, ECII may play a crucial role in the allosteric modulation of agonist binding by G proteins and GTP.

Recent elucidation of GPCR structures has provided extraordinary insight into the molecular details of receptor-ligand interaction as well as receptor G protein interaction (Cherezov et al., 2007; Rasmussen et al., 2007, 2011; Warne et al., 2008; Rosenbaum et al., 2011). Nonetheless, structural studies by their nature provide incomplete understanding of the basis of signal transduction. In general, the exofacial ligand-binding surface of the receptor does not appear to undergo large conformational changes upon binding an agonist as compared with the intracellular surface, which interacts and undergoes larger conformational changes, particularly of transmembrane helices V and IV (for review, see Lebon et al., 2012). Our data support the hypothesis that for the EP receptors ECII is an important sensor of agonist binding from the exofacial to intracellular surfaces. In the structures solved thus far, ECII has been found in a variety of conformations, including α-helices and β sheets. In some cases, ECII appears to make direct contact with bound ligands, whereas in other structures it does not (for review, see Peeters et al., 2011). It may be that, like the S1P receptor, the ECII of the lipid-ligated EP3 forms a tight lid over the ligand-binding pocket (Hanson et al., 2012). For the adenosine A2a receptor, there is evidence for direct ECII-agonist interaction, and significant movement of EC regions and transmembrane helices may transmit conformational changes that lead to signal transduction (Xu et al., 2011). Thus, there does not appear to be a universal role for ECII in receptor function, and structural studies will be required to understand the exact role of ECII in EP3.

Random mutagenesis of ECII in the C5a receptor has identified mutations that resulted in constitutively active receptors, consistent with a role for this region in interconversion of the R and R* states (Klo et al., 2005). It is interesting to note that random mutagenesis of the ECII region of the M3 muscarinic receptor has identified a class of mutations that were inactive or displayed reduced activity in signal transduction in intact cells, though they retained agonist binding in membrane preparations (Scarselli et al., 2007). Those investigators concluded that these mutations are important in signal transduction. In light of the findings described here, their results might also be consistent with alterations in GTP sensitivity of the agonist binding of the receptor. These mutations might then be unable to bind ligands in intact cells while retaining binding in broken-membrane preparations. It would be of interest to know whether they have altered GTP sensitivity in their ECII mutations of the M3 receptor as well.

Naturally occurring mutations in the EC loop regions of GPCRs may also affect ligand binding via directly perturbing...
the receptor–ligand interaction or by altering the coupled equilibrium interaction of the GPCR and the G protein. For example, naturally occurring mutations of the P2Y12 receptor have been described that affect activation but not ligand binding (Cattaneo, 2011). Mutations in the EC domains may play an important role in human disease.

The rabbit EP3 receptor displays high levels of constitutive activity, as has been observed for the EP3 receptor characterized in other species (Negishi et al., 1996; Hasegawa et al., 1997; Hitaka et al., 1997; Jinn et al., 1997). It has been suggested that the tight G protein binding is associated with both GTP-insensitive agonist binding as well as constitutive activity. Our results suggest that these phenomena are not necessarily linked, as the W203A mutation retains its constitutive activity despite its GTP-sensitive agonist binding. Constitutive activity has important implications in the physiology of the EP3 receptor. We and others have shown that the multiple PG receptors have functionally antagonistic actions. For example, the EP1 and EP3 receptors have pressor effects in vivo whereas the EP2 and EP4 receptors act as vasodepressors. Blockade of prostanoid biosynthesis would eliminate all ligand-evoked EP receptor signaling, but differences in constitutive activity of receptors may result in alterations in the balance of receptor action. A high level of EP3 constitutive activity in the absence of opposing EP2 and EP4 depressor effects might result in dominant EP3 effects, which would be expected to elevate blood pressure upon PG blockade by nonsteroidal anti-inflammatory drugs.

In summary, our study suggests that for the EP3 receptor, the ECII region can alter GTP effects on agonist binding and thus on intracellular signaling. These effects may be easily overlooked in systems where membrane fraction rather than intact cell binding is assessed. Because the ECII region is conserved among the eight PG receptors in this family, it seems plausible that this region may play a critical role for other members this family. Indeed, mutation of residues in the ECII region of the human EP2 and EP4 receptors leads to a complete loss of agonist binding in broken-cell membrane preparations (Stillman et al., 1998, 1999). It may also hold true for other family A GPCRs in general. These studies support a key role for the ECII region in triggering the conformational changes accompanying agonist-induced receptor activation and in determining the changes in receptor-agonist affinity in response to changes in the nucleotide bound state of the G protein in the cytoplasm.

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