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Protease-Activated Receptor 1 (PAR1) coupling to $G_{q/11}$ but not to $G_{i/o}$ or $G_{12/13}$ is mediated by discrete amino acids within the receptor second intracellular loop

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Abstract

Protease-activated receptor 1 (PAR1) is an unusual GPCR that interacts with multiple G protein subfamilies ($G_{q/11}$, $G_{i/o}$, and $G_{12/13}$) and their linked signaling pathways to regulate a broad range of pathophysiological processes. However, the molecular mechanisms whereby PAR1 interacts with multiple G proteins are not well understood. Whether PAR1 interacts with various G proteins at the same, different, or overlapping binding sites is not known. Here we investigated the functional and specific binding interactions between PAR1 and representative members of the $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ subfamilies. We report that $G_{q/11}$ physically and functionally interacts with specific amino acids within the second intracellular (i2) loop of PAR1. We identified five amino acids within the PAR1 i2 loop that, when mutated individually, each markedly reduced PAR1 activation of linked inositol phosphate formation in transfected COS-7 cells (functional PAR1-null cells). Among these mutations, only R205A completely abolished direct $G_{q/11}$ binding to PAR1 and also PAR1-directed inositol phosphate and calcium mobilization in COS-7 cells and PAR1^{−/−} primary astrocytes. In stark contrast, none of the PAR1 i2 loop mutations disrupted direct PAR1 binding to either G_o or G_{12} , or their functional coupling to linked pertussis toxin-sensitive ERK phosphorylation and C3 toxin-sensitive Rho activation, respectively. In astrocytes, our findings suggest that PAR1-directed calcium signaling involves a newly appreciated $G_{q/11}$ -PLC ϵ pathway. In summary, we have identified key molecular determinants for PAR1 interactions with $G_{q/11}$, and our findings support a model where $G_{q/11}$, $G_{i/o}$ or $G_{12/13}$ each bind to distinct sites within the cytoplasmic regions of PAR1.

Keywords

Protease-activated Receptor; PAR1; astrocytes; thrombin; G protein; G_q ; G_{11} ; G_o ; $G_{12/13}$; phospholipase C ϵ ; PLC ϵ

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1. Introduction

Protease-activated receptor 1 (PAR1) is a G protein-coupled receptor (GPCR) that was first identified as the thrombin receptor [1]. Although PAR1 is best known for its role in platelet activation and hemostasis [2], it also is expressed throughout the central nervous system (CNS) and has complex pathophysiological roles within the brain [3]. PAR1 activators are expressed in the brain parenchyma, and the receptor itself is expressed on both neurons and glia [4–6]. Under normal conditions, neuronal and astrocytic PAR1 is shielded from blood-borne proteases by the blood-brain barrier (BBB). However, following CNS injury such as in hemorrhagic stroke and subsequent BBB breakdown, high levels of these proteases infiltrate the brain tissue to regulate glial-mediated wound repair. Evidence indicates that brain-derived proteases such as plasmin can serve as endogenous agonists for PAR1 signaling to influence normal neuronal and glial physiology [7]. By acting on PAR1, the tPA/plasminogen/plasmin system acting on PAR1 has been shown to play important roles in the control of hippocampal neuronal plasticity, modification of the reward system, regulation of dopamine release from the nucleus accumbens, and CNS response to stress [8–13], among others. Activation of PAR1 may be either neuroprotective (i.e., by enhancing neuronal or astrocytic survival) or neurodegenerative (i.e., by regulating glutamate excitotoxicity and enhancing seizure sensitivity) [5, 14] depending on the concentration of protease. In summary, the CNS actions of PAR1 activation are vast and complex, though the molecular signaling events that underlie these functions have not yet been fully characterized.

PAR1 is one of four protease-activated receptors (PAR1–4) [1, 15–17] that are activated by serine proteases (e.g., thrombin, trypsin, plasmin and others) that cleave the receptor N-terminus. The newly unmasked extracellular N-terminus of PAR1 serves as an intramolecular ligand that activate the cleaved receptor to stimulate G proteins, which subsequently initiate a myriad of downstream signaling cascades [5, 18]. PARs are unconventional GPCRs that functionally interact with multiple G proteins, with PAR1 capable of physically coupling to individual members of the $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ subfamilies [5, 18, 19]. In doing so, PAR1 also activates multiple G protein-linked effector pathways in most cell types including mitogen-activated protein kinase (MAPK), various Rho kinase and phospholipase C (PLC) isoforms, inositol lipid signaling, and mobilization of intracellular calcium [5, 18, 20]. The capacity to activate multiple G proteins is unusual among GPCRs, making the understanding of PAR1/G protein coupling a particularly important mechanism for investigation. Whether members of different G protein subfamilies bind to PAR1 simultaneously or individually, and whether these G proteins have distinct or overlapping binding sites on PAR1 is not known.

Several studies have sought to identify domains on PAR1 responsible for G protein coupling [21, 22]. Prior experiments of chimeric receptors that transferred the cytoplasmic portions of PAR1 into either the G_s -linked β_2 -adrenergic receptor (β_2AR) or the G_i -linked dopamine D_2 receptor (D_2R) determined that the second intracellular loop (PAR1 i2 loop) of PAR1 is responsible for receptor coupling to $G_{q/11}$, leading to inositol phosphate (InsP) formation, intracellular calcium mobilization, and related signaling [22]. Subsequent studies implicated key roles for the PAR1 second intracellular (i2) and third intracellular (i3) loops in PAR1/G protein coupling by using cell-penetrating, membrane-tethered peptides corresponding to these domains to prevent PAR1 activation of calcium signaling [23]. Using molecular modeling approaches, a more recent study proposed that coordinated actions among different domains are required for G protein coupling to PAR1. Specific domains included the PAR1 seventh transmembrane domain, eighth helix (part of the receptor's cytoplasmic tail), and the i1 loop [21]. Together, these studies broadly identified PAR1 domains responsible for activation of G protein signaling pathways located within the receptor's

intracellular loops and cytoplasmic tail. However, uncoupling the binding and functioning of one G protein to PAR1 activation, while preserving the binding and functioning of other G proteins has not yet been demonstrated.

Based on previous studies implicating the i2 loop of PAR1 in $G_{q/11}$ coupling [22], we investigated direct and functional G protein coupling to PAR1 with the goal of determining: 1) whether different G proteins can bind to overlapping sites within the i2 loop, and 2) can PAR1 binding to one G protein be selectively uncoupled while preserving PAR1 binding to other G proteins. We employed alanine-scanning mutagenesis to create a series of 21 individual receptor mutants, each containing a different single point mutation within the PAR1 i2 loop. Each mutant was then tested for physical and functional coupling to different G proteins and linked signaling pathways. We have identified five amino acids located within the i2 loop of PAR1 that contribute to PAR1/ $G_{q/11}$ coupling, and a single amino acid (Arg205) that, when mutated, disrupts direct PAR1 binding to and functional coupling with $G_{q/11}$ but which has no effect on PAR1 coupling to either $G_{i/o}$ or $G_{12/13}$. Our findings highlight a previously unknown mechanism of PAR1/G protein coupling, suggesting that G protein subfamily members rely on distinct amino acids and binding sites for functional coupling to the receptor.

2. Experimental procedures

2.1. Materials

Materials and reagents used in our studies were purchased from the following sources: QuikChange mutagenesis kit from Stratagene (La Jolla, CA); Anti-FLAG M2 affinity gel, bovine serum albumin (BSA), U73122, penicillin, and streptomycin from Sigma Chemical Co. (St. Louis, MO); fetal bovine serum (FBS) from Atlanta Biologicals (Atlanta, GA); trypsin, Dulbecco's modified Eagle's medium (DMEM) from Cellgro (Herndon, VA). Lipofectamine 2000 and Fura-2, AM were obtained from Invitrogen (Carlsbad, CA); *myo*-[3 H]inositol from American Radiolabeled Chemicals, Inc. (St. Louis, MO); RhoA G-LISA™ Activation Assay colorimetric format kit and C3 exoenzyme from Cytoskeleton, Inc. (Denver, CO); Pertussis toxin from List Biologicals (Campbell, CA); p44/42 ERK1/2 (extracellular signal-regulated kinase 1/2) antibody and phospho-p44/42 ERK1/2 antibody from Cell Signaling Technology (Beverly, MA); anti- G_{α_o} and anti- $G_{\alpha_{12}}$ antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti- $G_{\alpha_{q/11/14}}$ antibody, Z811, was kindly provided by Dr. Paul Sternweis (U. Texas Southwestern, Dallas, TX); peroxidase-conjugated goat anti-rabbit was from Bio-Rad (Hercules, CA); and Amaxa® mouse astrocyte nucleofactor® kit (Lonza, Köln, Germany). The PAR1-activating peptide TFLLR-NH₂ (TFLLR) was synthesized by Dr. Jan Pohl at the Emory University Microchemical Facility (Atlanta, GA) or GenScript (Piscataway, NJ).

2.2. Alanine scanning mutagenesis

To introduce site-specific mutations into the mouse mPAR1-mCherry-FLAG clone, the QuikChange mutagenesis kit was used according to the manufacturer's suggestions. Alanine-scanning mutagenesis was carried out to create a series of 21 mutations in the i2 loop of PAR1. Mutant receptors were generated during separate site-directed mutagenesis reactions with different primer sets, each using the original wild type mPAR1-mCherry-FLAG clone as a template. To confirm that the appropriate amino acid was changed to an alanine residue, cDNA sequencing was performed by Agencourt (Beverly, MA).

2.3. COS-7 cell cultures and transfection

COS-7 cells (ATCC® Number CRL-1651™) were cultured in DMEM with sodium pyruvate supplemented with heat inactivated FBS, 100 μ g/mL streptomycin and 100U/mL penicillin

at 37°C in a humidified atmosphere with 5% CO₂. Subculturing of confluent plates was done at a ratio of 1:10 for transfection. COS-7 cells were transfected according to the Lipofectamine 2000[®] transfection reagent protocol and cells were used for experimentation 24–48 h after transfection. For the time-course InsP accumulation assays, transfections were performed using previously described protocols with polyethyleneimine (PEI; Polysciences, Inc.) [24].

2.4. Animals

PAR1^{−/−} and wild-type (wt) mice were created as described [25]. Briefly, we bred *PAR1*^{+/−} mice, a generous gift from Dr. Shaun Coughlin (University of California, San Francisco, CA), with wt C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbor, ME). We bred heterozygous littermates to generate homozygous null mutants and wt controls that were > 99% C57BL/6. All procedures using animals were approved by the Emory University Institutional Animal Care and Use Committee.

2.5. Astrocyte cultures and transfections

Cultured astrocytes were prepared from P0–P3 postnatal *PAR1*^{−/−} mice; all protocols involving the use of animals have been reviewed and approved by the Emory University IACUC. After removing the meninges, the cortex was dissected out, pipetted up and down to form a single-cell suspension, and plated in T-75 flasks with DMEM containing 4.5 g/L glucose, 10 % each heat-inactivated horse serum and FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin. Transfection was performed 7–8 days later using an Amaxa[®] mouse astrocyte nucleofection[®] kit according to the manufacturer's instructions. Briefly, astrocytes were dissociated from the plate with 0.25% trypsin/EDTA and resuspended in nucleofector solution. After addition of 2 µg plasmid DNA per transfection, the cells were nucleofected with an Amaxa Nucleofector[®] II device. After allowing the nucleofected cells to recover in culture medium, they were plated in 12-well plates containing microscope coverslips coated with 0.05 mg/ml poly-D-lysine for imaging experiments or coated plates for InsP accumulation experiments. Transfections for each construct were performed at least twice with animals from different litters.

2.6. Knock-down of PLCε in astrocytes by siRNA Treatment

Mouse primary astrocytes were prepared from *PAR1*^{−/−} mice as described above. Seven days later, the cells were nucleofected with wild type *PAR1* or *PAR1*-R205A using 2×10⁶ cells and 2 µg plasmid DNA per nucleofection. The cells were plated in 12-well plates containing poly-D-lysine-coated coverslips, and incubated at 37°C with 5% CO₂ for 2 days. For siRNA treatment, the cells were washed once with HBSS and treated with adenovirus containing PLCε or random-sequence (control) siRNA at 100 multiplicity of infection (MOI) for 4 hr as described [26]. Following another wash to remove any extraneous virus, the cells were incubated at 37°C/5%CO₂ for 3 days before calcium imaging. The efficiency of PLCε knock-down was determined by reverse transcriptase-PCR (RT-PCR). Total RNA was isolated from siRNA-treated astrocytes using the PureLink[™] RNA mini Kit (Ambion) according to the manufacturer's instructions. RT-PCR was carried out with 50 ng total RNA as starting template using the SuperScript[™] III One-Step RT-PCR System with Platinum[®] Taq DNA Polymerase (Invitrogen) according to the manufacturer's instructions. Primer sequences were (5'→3'): PLCε: ACC CTG CGG TAA ATG TTC TG and ATG TGA ATT CCG TGC TAC CC [27]; β-actin: TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA and CTA GAA GCA TTG CGG TGG ACG ATG GAG GG [28]. Amplification was carried out at a melting temperature of 60°C for 35 cycles.

2.7. Immunoblot Analysis

Nitrocellulose membranes were incubated in blocking buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5% dried milk, 0.5% Tween 20, 0.02% sodium azide) at room temperature for up to 1 h and then were placed in a dilution of the indicated primary antibody at room temperature for 3 h or overnight at 4°C. Dilutions for each antibody differed: anti-mCherry 1:1000, anti-p44/42 ERK1/2 1:300 and anti-phospho p44/42 1:1000 in Tris-buffered saline + 0.1% Tween 20 (TBST) with BSA; anti-Gα_q family Z811 1:1000, anti-Gα_o 1:200; anti-Gα₁₂ 1:200 in blocking buffer. After being washed three times with TBST, membranes were probed with horseradish peroxidase-conjugated goat-anti-rabbit IgG at 1:25,000 in TBST for 1 h, room temperature. Protein were visualized using enhanced chemiluminescence (ECL).

2.8. Measurement of [³H]InsP formation

Levels of [³H]inositol phosphate ([³H]InsP) accumulation were determined in confluent plates of COS-7 cells or astrocytes from PAR1^{-/-} mice transiently transfected with pcDNA3.1, Flag-M1-muscarinic cholinergic receptor (kindly provided by Dr. Randy Hall), mouse PAR1-mCherry-Flag/pcDNA3.1, or the same PAR1 construct containing single i2 loop mutants. After a 5 hour (for Lipofectamine 2000[®]) or 24 hour (for PEI) transfection period for COS-7 cells or 2 days for primary astrocytes, cells were metabolically labeled with *myo*-[³H]inositol in serum-free media for 18–24 h. Prior to experimentation, the pharmacological inhibitor of PLC signaling, U73122, was added for 30 min in incubation buffer (DMEM buffered with 25 mM HEPES, pH 8.0, and containing 10 mM LiCl₂), where indicated. Then 30 μM TFLLR was used to stimulate the cells for either 0 sec, 10 sec, 30 sec, or 30 min. To stop the reactions, cells were solubilized with 20 mM formic acid and subsequently neutralized with 0.7 M NH₄OH. [³H]InsPs were subjected to anion exchange chromatography (AG 1-X8 Dowex, Bio-Rad) to isolate [³H]InsPs, which were quantified by scintillation counting and expressed as mean ± S.E.M.

2.9. Measurement of ERK1/2 phosphorylation

After serum starvation in the absence or presence of pertussis toxin (PTX) overnight, COS-7 cells separately transfected with empty vector control, or vector carrying wild type mPAR1, or mutated mPAR1 were stimulated with 30 μM TFLLR for 5 min, harvested in Laemmli Sample Buffer, sonicated on ice, boiled, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 13.5%). Samples then were transferred to nitrocellulose membranes for immunoblotting with p44/42 ERK1/2 and phospho- p44/42 ERK1/2 antibodies.

2.10. Measurement of RhoA activation

Activated RhoA was measured with an absorbance-based RhoA Activation G-LISA[™] kit (Cytoskeleton, Inc., Denver, CO) by following the manufacturer's protocol. Transfected COS-7 cells were serum-starved overnight and then treated with C3 toxin for 4 h, where indicated. A 30 sec addition of 30 μM TFLLR was used to elicit the Rho response. The absorbance from the G-LISA[™] plate was read by a spectrophotometer at wavelength 490nm.

2.11. Co-Immunoprecipitation of PAR1/G protein complexes

Immunoprecipitation of mPAR1 was performed as described previously [19]. Briefly, COS-7 cells were transiently transfected for 18–24 h with PAR1/ G protein pairs containing either wild type or mutant PAR1 and individual G proteins, as indicated. Unstimulated cells were washed once in PBS and harvested in 0.5 ml of Tris Buffer (50 mM Tris, pH 7.4, 5 mM MgCl₂, 1 mM EGTA, 150 mM NaCl, 1 mM EDTA, plus a protease inhibitor tablet (Roche)), and sonicated on ice. The detergent *n*-Dodecyl-β-D-maltoside (DβM; Calbiochem)

was added to a final concentration of 2% for 3 h rotating end-over-end at 4°C for membrane protein extraction. Debris was then pelleted by ultracentrifugation (100,000 × g, 4°C, 30 min). Inputs shown are an aliquot of the lysates from just after the ultracentrifugation step. The remaining cytosol was rotated end-over-end overnight at 4°C with pre-blocked anti-FLAG M2 affinity gel. The anti-FLAG resin was pelleted the following day, washed with Tris Buffer containing 0.2% DβM, and resuspended in 2X Laemmli Sample Buffer. Supernatants were loaded onto 11% polyacrylamide gels for SDS-PAGE separation, transferred to nitrocellulose membranes, and immunoblotted as described.

2.12. Calcium Imaging

Calcium imaging of cultured astrocytes was performed as described [29]. Briefly, cells were incubated in 5 μM Fura-2, AM in 0.1% Pluronic® F-127 (Invitrogen) for 30–40 min at 37°C, after which coverslips were transferred to a microscope stage for imaging. The external buffer contained (mM) 150 NaCl, 10 Hepes, 3 KCl, 2 CaCl₂, 1 MgCl₂, 22 sucrose, and 5.5 glucose. Buffer pH was adjusted to 7.4. Imaging was performed at room temperature (23°C) with dual excitation at 340 nm and 380 nm wavelengths using a MicroMax Camera (Princeton Scientific Instruments, Inc., Monmouth, NJ), and the two resulting images were used for ratio calculations (Ex 340/Ex 380) using Axon Imaging Workbench version 6.0 (Axon Instruments). The imaging protocol consisted of baseline reading for 60 s, application of 50 μM TFLLR for 60 s, wash for 120 s, application of 30 μM ATP for 30 s (positive control), and final wash for 120 s. In order to facilitate comparisons between cells with different background levels of free calcium, all calculated ratios were normalized to the

baseline reading according to the formula $R_x(t) = \frac{R_x(t)}{\text{avg}(R(60):R(0))}$, where $R(t)$ is the normalized ratio, $R_x(t)$ is the raw ratio at each time point, and $\text{avg}(R(60):R(0))$ is the average of the raw ratios in the first 60 seconds before cells were stimulated with TFLLR.

2.13. Immunofluorescence and confocal imaging

Following nucleofection, astrocytes were fixed at room temperature for 15 mins in buffer containing 3.7% paraformaldehyde diluted in PBS. Cells were washed in PBS and incubated for 8 mins with 0.4% TritonX-100 diluted in PBS. Cells were washed with PBS again (3X) and mounted with ProLong Gold Antifade Reagent (Invitrogen). Confocal images were taken using a 63× oil immersion objective from a LSM510 laser scanning microscope with AxioObserver Stand (Zeiss). Images were processed using the ZEN 2009 Light Edition software and Adobe Photoshop 7.0 (Adobe Systems).

3. Results

3.1. Five amino acid residues in the PAR1-i2 loop are important for the receptor's capacity to activate inositol phosphate signaling

PAR1 stimulates one or more isoforms of phospholipase C (PLC) to initiate phosphatidylinositol (4,5)-bisphosphate hydrolysis and InsP signaling [20, 30, 31]. Evidence suggests that the i2 loop of PAR1 alone is sufficient to confer capacity for G_{q/11} coupling (InsP signaling) to different receptors that exclusively link to G_{i/o} or G_s (i.e., D₂R and β₂-AR, respectively) [22]. However, the precise sites within this cytoplasmic domain that are important for this signaling event to occur have not been identified. The PAR1 i2 loop contains 21 amino acids. Therefore, we created a set of 21 distinct mutant PAR1 receptors, each with a discrete alanine substitution point mutation for the individual amino acid in the receptor's i2 loop, and evaluated their capacity to stimulate InsP signaling (Figure 1). These assays were performed by expressing each recombinant PAR1 mutant in COS-7 cells. Of the 21 mutants we screened, all but three (I203A, D204A, and R219A) express at levels

detectable by western blotting and comparable to wild type PAR1 (Figure 1). Therefore, we excluded these three poorly expressing receptor mutants from our remaining studies and proceeded to assess function of the other 18 mutant receptors.

COS-7 cells have been reported to express undetectable levels of PAR1 [15, 32]. We recently confirmed and expanded on these findings by using COS-7 cells as a functional PAR1-null cell system to show that PAR1 directly binds and functionally couples to various members of the $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ subfamilies of G proteins [19]. Consistent with earlier reports, we found that COS-7 cells did not stimulate InsP signaling in response to the selective PAR1 agonist TFLLR, as is shown in our pcDNA3.1 vector only controls (Figure 1). To determine whether any of the PAR1 i2 loop mutants had reduced capacities to stimulate PLC- β activity, we compared accumulation of InsPs in cells transiently transfected with either wild type PAR1 or the mutant receptors and activated with TFLLR (Figure 1). From this screen, we identified five residues (R205A, V209A, P212A, I213A, and L216A) at which substitution of an alanine reduces InsP signaling relative to wild type recombinant PAR1 by more than 70% (dotted line). These data identify individual candidate amino acid residues within the i2 loop of PAR1 that contribute to its functional coupling to $G_{q/11}$ -PLC-mediated InsP signaling in COS-7 cells.

3.2. PAR1 mutants that disrupt $G_{q/11}$ coupling do not affect PAR1 coupling to $G_{i/o}$ or $G_{12/13}$

Our recent work showed that PAR1 couples to $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ to initiate signaling pathways linked to these G protein families [19]. To explore whether the five amino acid residues identified in our screen (Figure 1) are also important for $G_{i/o}$ and $G_{12/13}$ coupling, we tested the capacities of these five PAR1 i2 loop mutants to activate $G_{i/o}$ - and $G_{12/13}$ -linked signaling pathways. As before (Figure 1), wild type PAR1 and all of the mutant receptors, but not the pcDNA3.1 vector control, are detected upon immunoblotting with the anti-mCherry antibody (Figure 2A). We then sought to determine the relative capacities of these mutant PAR1 receptors to couple to $G_{12/13}$ and $G_{i/o}$, which link PAR1 to activation of RhoA and Erk, respectively, in COS-7 cells, which we have shown previously [19] (Figure 2A–C). To confirm that the functional readouts of InsP accumulation, Rho signaling, and ERK1/2 phosphorylation reflect true measures of the linked G proteins $G_{q/11}$, $G_{12/13}$, and $G_{i/o}$, we employed selective pharmacological inhibitors of these pathways (i.e., U73122, C3 toxin, and PTX, respectively) to determine their effects on the signalling activities of wild type PAR1 and the PAR1 mutant receptors' signaling activities (Figure 2A–C).

We first confirmed that activation of inositol lipid signaling by wild type PAR1 and the PAR1 i2 loop mutants is mediated by $G_{q/11}$ in COS-7 cells (Figure 2A). Because both $G_{q/11}$ and $G_{12/13}$ can activate InsP signaling through two isoforms of PLC, PLC- β and PLC- ϵ , we used a known PLC inhibitor (U73122) to test whether residual InsP production is mediated through one of these PLC isoforms. We found that wild type PAR1-stimulated InsP production is reduced by nearly 75% and that the residual signaling associated with the five PAR1 i2 loop mutants is reduced to control levels in the presence of U73122 (Figure 2A). These data suggest that wild type PAR1 and PAR1 i2 loop mutant receptors stimulate InsP production by activation of $G_{q/11}$ /PLC under these experimental conditions.

We next tested (Figure 2B) whether these PAR1 mutants couple to $G_{12/13}$, the G protein subfamily primarily responsible for GPCR-stimulated Rho signaling [33–35]. We recently showed that PAR1 stimulated RhoA signaling in COS-7 cells, mediated most likely by $G_{12/13}$ activation of p115RhoGEF [19] or by $G_{q/11}$ stimulation of p63RhoGEF [36, 37]. We thus determined whether the PAR1 i2 loop mutants retained capacities to stimulate RhoA activation (as indicated by RhoA-GTP formation) in these cells. We found that all of the receptors with mutations that blocked PAR1-mediated InsP signalling still triggered RhoA activation at levels comparable to that of wild type PAR1 (Figure 2B), and that this activity

was inhibited by C3 toxin, a pharmacological inhibitor of RhoA. These results indicate that all of the mutant receptors identified in our InsP screen are otherwise functional and retain full capacities to activate RhoA signaling, most likely via $G_{12/13}$ -linked activation of p115-RhoGEF. These results also suggest that none of the five amino acid residues are essential for $G_{12/13}$ coupling to PAR1 in these cells using these methods.

We also tested these mutants for their capacities to couple to $G_{i/o}$ which we show is largely responsible for PAR1 activation of ERK/MAP kinase (MAPK) signaling in COS-7 cells [19]. We investigated the capacities of the mutant PAR1 receptors to activate PAR1/ $G_{i/o}$ -directed stimulation of ERK1/2 phosphorylation (Figure 2C). We found that, similar to our RhoA activation experiments, all of the tested PAR1 i2 loop mutant receptors retain full capacity to stimulate ERK1/2 phosphorylation. The ERK signaling response by both wild type PAR1 and mutant receptors was markedly reduced by pretreatment with pertussis toxin (PTX), a specific inhibitor of receptor-activated $G_{i/o}$ signaling. These data indicate that the five PAR1 mutants tested retain full capacities to signal through $G_{i/o}$ -linked ERK/MAPK pathways, suggesting that the amino acids in the PAR1 i2 loop that control $G_{q/11}$ signalling do not dictate $G_{i/o}$ coupling to PAR1 under these experimental conditions.

3.3. Two PAR1 mutant receptors have greatly reduced capacities to directly bind G_{11} , but not to G_o or G_{12}

After demonstrating that the five identified mutant PAR1 receptors can functionally uncouple PAR1 from $G_{q/11}$, but not $G_{i/o}$ - or $G_{12/13}$ -mediated signaling, we determined the relative capacities of these mutated receptors to form stable complexes with individual G protein $G\alpha$ subunits. Using immunoprecipitation techniques, we previously showed that PAR1 forms stable complexes with various individual members of the $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$ subfamilies [19] including but not limited to $G\alpha_{11}$, $G\alpha_o$ and $G\alpha_{12}$. Here, we used the same technique to evaluate the relative capacities of the PAR1 i2 loop mutant receptors to interact with representative $G\alpha$ subunits from each of these disparate subfamilies ($G\alpha_{11}$, $G\alpha_o$ and $G\alpha_{12}$) (Figure 3A). Empty vector control, FLAG-tagged wild type PAR1 or FLAG-tagged PAR1 mutant receptors and individual $G\alpha$ protein subunits were co-transfected into COS-7 cells. Anti-FLAG affinity resin was used to recover the PAR1/ $G\alpha$ complexes and recovered proteins were detected by immunoblotting (Figure 3A, top). As expected, no G proteins were recovered when the individual G proteins were co-expressed and recovered by immunoprecipitation with the control vector (Figure 3A, lane 1). By contrast, all of the tested G proteins ($G\alpha_{11}$, $G\alpha_o$ and $G\alpha_{12}$) formed stable complexes with wtPAR1 (Figure 3A), as previously shown [19]. However, two of the tested PAR1 i2 loop mutants, R205A and L216A, had markedly reduced capacities to interact with $G\alpha_{11}$ (Figure 3A), while all of the other mutant receptors retained capacities to bind $G\alpha_{11}$ (Figure 3A). Furthermore, all of the tested receptor mutants (including R205A and L216A) retained capacity to form complexes with $G\alpha_o$ and $G\alpha_{12}$ (Figure 3A).

Since PAR1 mutants R205A and L216A exhibited reduced $G\alpha_{11}$ binding, we further examined the functional capacity of these two mutant receptor constructs to stimulate InsP accumulation in COS-7 cells (Figure 3B) mediated by one or more PLC isoforms (Figure 2A). When examined after short (0–30 sec) or long (up to 30 min) periods of stimulation, we found that L216A remains functionally coupled to $G\alpha_{q/11}$ and PLC activation (albeit weakly), whereas R205A does not. The PAR1 R216A mutant stimulated InsP to levels approximately 40% that of wild type PAR1 (Figure 3C), indicating that while R216A does not form a stable complex with $G\alpha_{11}$, it retains capacity to activate the G protein and downstream signalling. Taken together, these data (Figures 2 and 3) show that discrete amino acids in the i2 loop of PAR1, particularly R205, control receptor binding and functional coupling to $G\alpha_{11}$, but are not necessary for binding to and activation of $G\alpha_o$ or $G\alpha_{12}$.

3.4. PAR1 i2 loop mutants have differential capacities to stimulate InsP accumulation and calcium signaling in astrocytes from PAR1^{-/-} mice

For the experiments described up to this point, we have used functional PAR1-null cells (COS-7 cells) to compare the G protein coupling capacities of PAR1 i2 loop mutant receptors with wild type PAR1/G protein coupling. These studies (Figures 1–3) have identified five amino acid residues (R205A, V209A, P212A, I213A, and L216A) that contribute to functional coupling of PAR1 to G₁₁ but not to G_o or G₁₂. Of these five residues, only R205A completely uncouples PAR1 from direct binding and functional interactions with G_{q/11} and activation of PLC. To further explore the physiological relevance of these findings, we shifted our focus from the COS-7 cell line to cultured primary astrocytes derived from PAR1^{-/-} mice, natural host cells for PAR1 and therefore a more physiologically relevant system.

Our previous studies reported that activated wild type PAR1 stimulates measurable increases in intracellular calcium levels in primary cultures of astrocytes from wild type mice through a PLC-mediated (U73122-sensitive) signaling pathway [29]. We also demonstrated that astrocytes from PAR1^{-/-} mice do not mobilize intracellular calcium in response to TFLLR agonism [29], as expected. Therefore, we used this system as a means to further characterize the G protein coupling capacities of mutant PAR1 receptors (Figure 4). Astrocytes expressing recombinant wild type PAR1 invoke measurable increases in intracellular calcium concentrations relative to cells expressing vector alone. As is shown in the vector only controls, TFLLR has no effect in astrocytes from PAR1^{-/-} mice (Figure 4) under conditions whereas ATP activation of an endogenous G_{q/11}-linked purinergic receptor elicited calcium mobilization. By contrast, in cells expressing wild type PAR1, TFLLR elicits a readily measurable increase in intracellular calcium response (Figure 4). These data are consistent with several studies that have previously demonstrated the positive effects of thrombin (i.e., mediated by PAR1 and potentially other PARs) on calcium signaling in glial cells [29, 38–42]. Similar to wild type PAR1, the receptor mutants V209A, P212A, I213A, and L216A each exhibited a capacity to stimulate increases in calcium levels when activated by TFLLR (Figure 4). However, the R205A mutant receptor alone completely lost capacity to initiate calcium mobilization in response to TFLLR (Figure 4), suggesting that this arginine residue is necessary for PAR1/G_{q/11}-mediated signalling responses in these cells using these methods. As a control for cell viability, all cells expressing PAR1 mutants retained capacity to mobilize calcium in response to the endogenous G_{q/11}-linked ATP/purinergic receptor (Figure 4). Of these five mutants, only the R205A mutant of PAR1 lost capacity to bind G₁₁ in co-IP experiments and to stimulate PLC (Figure 3). Taken together, these findings indicate that although five point mutations in the PAR1 i2 loop reduce the receptor's capacity to couple to G_{q/11}-linked pathways, only one, R205A, completely abolishes both direct PAR1/G₁₁ binding as well as linked calcium signalling in response to PAR1 agonist.

A previous report [43] indicates that PAR1 coupling to InsP accumulation and calcium signaling in astrocytes is mediated, at least in part, by indirect activation of PLC ϵ . As such, we next tested the capacity of PAR1 and receptor mutant R205A to stimulate InsP accumulation (Figure 5) and calcium mobilization in astrocytes, and the effects of knocking down PLC ϵ on this calcium signal (Figure 6). To confirm that the R205A mutant of PAR1 was expressed in the cultured PAR1^{-/-} astrocytes (Figure 5), cells were again transfected by nucleofection with cDNA encoding either the empty vector (control), wild type PAR1, or the mutated PAR1-R205A. Transfected cells then were either radiolabelled with [³H]myo-inositol and stimulated with TFLLR (Figure 5B) or fixed for fluorescence (mCherry) confocal imaging (Figure 5A). Both the recombinant wtPAR1-mCherry and PAR1-R205A-mCherry were expressed in PAR1^{-/-} astrocytes to similar levels and with a similar subcellular distribution pattern. Consistent with what we observed with COS-7 cells

(Figures 1, 2B and 3B), activation of wild type PAR1 stimulated InsP accumulation whereas activation of PAR1-R205A did not (Figure 5).

We next tested the relative contribution of PLC ϵ to the PAR1-stimulated calcium signal in PAR1 $^{-/-}$ astrocytes (Figure 6). We found that cells pretreated with siRNA targeted specifically at PLC ϵ [26, 44], but not randomly scrambled control siRNA, markedly reduced PLC ϵ mRNA levels by approximately 80%. When these cells were also transfected to express either wild type PAR1 or PAR1-R205A, the TFLLR-induced calcium response was reduced by approximately 40% (Figure 6F), which is consistent with an earlier report suggesting that a large portion of the PAR1 calcium signal in astrocytes is mediated by indirect activation of PLC ϵ via a RhoA-GTP signaling pathway [43]. As before, stimulation of R205A failed to mobilize calcium. Of note, ATP activation of endogenous purinergic receptors also stimulated calcium signaling, and this also was partially reduced (60%) by knock-down of PLC ϵ . Taken together, these data (Figures 5 and 6) indicate that PAR1 coupling to G $_{q/11}$ and downstream InsP and calcium signaling in astrocytes is completely abolished when residue R205 within the i2 loop of the receptor is mutated to alanine (R205A).

4. Discussion

Well-established models of GPCR/G protein coupling based on decades of research propose that receptors link to a single G protein, which interacts directly with one or more cytoplasmic domains of the receptor typically involving the i2 loop, the i3 loop, the C-terminal tail, or some combination of these. PAR1 and a limited number of other identified GPCRs diverge from this model in that they functionally couple to multiple G proteins [45, 46], and recent reports suggest that the i2 loop of PAR1 is primarily responsible for functional G $_{q/11}$ coupling [22]. Given this information, the primary goals of these studies were to determine if different G proteins couple to the same, different or overlapping sites on the i2 loop of PAR1, and whether discrete sites could be identified on PAR1 that functionally uncouple G $_{q/11}$ signaling from G $_{i/o}$ and G $_{12/13}$ signaling. Our key findings show that: 1) five discrete amino acids within the PAR1 i2 loop (R205A, V209A, P212A, I213A and L216A) reduce capacity of the receptor to stimulate G $_{q/11}$ /PLC-mediated inositol lipid signaling; 2) mutating these five amino acids within the i2 loop has no effect on PAR1 coupling to G $_{12/13}$ -mediated RhoA activation or G $_{i/o}$ -stimulated ERK1/2 phosphorylation; 3) two of these mutations (R205A and L216A) limit PAR1 and G $_{11}$ from forming a stable complex; and 4) of these two mutants, only R205A disrupts stable PAR1/G $_{11}$ complex formation and also downstream InsP formation and calcium mobilization in COS-7 cells and in host astrocytes from PAR1 $^{-/-}$ mice. We will discuss each of these findings.

4.1. Five discrete point mutations within the PAR1 i2 loop reduce the capacity of the receptor to stimulate G $_{q/11}$ /PLC- β -mediated inositol lipid signaling

The i2 loop of the PAR1 receptor contains 21 amino acids. Our findings (Figure 1) indicate that five of these amino acids contribute to functional G $_{q/11}$ coupling and InsP production stimulated by PAR1. Our studies focused specifically on the PAR1 i2 loop since earlier work showed that this domain was sufficient to invoke G $_{q/11}$ -like coupling and inositol lipid signaling when introduced into the G $_s$ -linked β_2 -AR and the G $_{i/o}$ -linked D $_2$ R [22]. By contrast, neither the PAR1 i1 loop or i3 loop conferred this signaling capacity on non-G $_{q/11}$ -linked receptors. Of note, both the extreme N- and C-terminal portions of the PAR1 i2 loop were necessary for these chimeric receptors to initiate InsP signaling [22]. Consistent with these findings, the five point mutations that we identified to contribute to PAR1/G $_{q/11}$ coupling in our screen span both regions of the i2 loop. Our findings show that discrete amino acids within the i2 loop are important for PAR1/G $_{q/11}$ functional coupling, but do not necessarily rule out a contributing role for the receptor i3 loop and the C-tail in this process.

4.2. The five PAR1 i2 loop point mutations that disrupt InsP signaling have no effect on G_{12/13}-mediated RhoA activation or G_{i/o}-stimulated ERK1/2 phosphorylation

PAR1 and a limited number of other GPCRs are known to functionally couple to multiple G proteins [[45, 46] (and references therein)]. Previous studies have attempted to define the molecular determinants for such “promiscuous” receptor/G protein couplings. Evidence suggests that altering distinct regions on several of these receptors (e.g. α_2A -adrenergic, calcitonin, cholecystokinin, endothelin, glutamate mGluR1, and others) disrupts activation of certain G protein pathways (reviewed in [45]). Consistent with these reports, our results indicate that the five point mutations that reduce the coupling efficacy of PAR1 and G_{q/11} have no effect on G_{12/13}- or G_{i/o}-linked signaling pathways when assessed as PAR1-mediated stimulation of C3 toxin-sensitive RhoA-GTP formation or PTX-sensitive ERK1/2 phosphorylation, respectively (Figure 2C–D). Significantly, all of the PAR1 i2 loop mutants tested retained full capacities to activate both G proteins and their linked signaling pathways, suggesting that other receptor regions are most important for PAR1 coupling to G_{i/o} and G_{12/13}. The cytoplasmic loop regions of PAR1 are relatively small compared to other GPCRs. As such, spatial and/or steric constraints likely preclude the simultaneous binding of multiple G proteins to the i2 loop. Therefore, our findings support the idea that PAR1 binding sites for the G_{q/11} subfamily likely differ from the binding sites for G_{12/13} and G_{i/o} subfamily members.

4.3. Of the five point mutations that inhibit PAR1/G_{q/11} functional coupling, only two limit PAR1 and G₁₁ from forming a stable complex

We recently demonstrated that PAR1 forms stable complexes with multiple G protein heterotrimers (i.e., G $\alpha_{11}\beta\gamma$ as well as G $\alpha_o\beta\gamma$) [19]. Here, we sought to determine whether these interactions would be disrupted by our identified PAR1 i2 loop point mutations. We found that all of the PAR1 i2 loop mutants tested retain capacity for direct binding to G α_{12} and G α_o (Figure 3) as well as linked RhoA and ERK signaling, respectively (Figure 2C–D). These findings are consistent with the idea that G α_{12} and G α_o each bind to and functionally couple to sites or regions on PAR1 beyond the i2 loop. By contrast, only two of the five mutants that had reduced coupling to G_{q/11}-PLC-InsP signaling (R205A and L216A) showed reduced capacity for direct binding to G₁₁. These findings were somewhat unexpected since loss of signalling function would be expected to correlate with loss of G protein binding capacity. However, the immunoaffinity trap assays used to recover the stable PAR1/G protein complexes expose the proteins to relatively harsh conditions that only higher affinity protein interactions would survive. A likely possibility is that PAR1 mutants V209A, P212A and I213A each retain capacity for high affinity G_{q/11} binding and physical interactions, but have reduced capacity for signal transfer between receptor and G_{q/11}. By contrast, PAR1 mutant L216A apparently has reduced binding affinity for G₁₁ and a diminished but still intact capacity for signal transfer to G_{q/11}, whereas R205A exhibits a complete loss of G_{q/11} binding combined with a complete loss of functional coupling.

4.4. A single point mutation in the PAR1 i2 loop (R205A) disrupts downstream InsP signaling and calcium mobilization in astrocytes from PAR1^{−/−} mice

We also tested the signaling properties of PAR1 i2 loop mutants in primary astrocytes, natural host cells for PAR1 that lack the PAR1 gene (PAR1^{−/−}). Of the five mutants tested, only one (R205A) lost capacity to stimulate both InsP accumulation and calcium mobilization relative to wild type PAR1 (Figures 4–6). These findings are consistent with our results in COS-7 cells (Figures 1–3). While the capacity of mutants V209A, P212A, I213A and L216A for stimulation of InsP is reduced, coupling to G_{q/11} is still sufficient to mobilize calcium (Figure 4). In stark contrast, coupling of PAR1 mutant R205A to G_{q/11} and linked InsP/calcium signaling is eliminated entirely (Figures 4–6). Of note, activated G $\alpha_{q/11}$ can directly stimulate both PLC β and p63RhoGEF [37, 47]. Our findings (Figure 6) indicate

that PAR1- $G_{q/11}$ -directed activation of InsP and calcium signaling in astrocytes is dependent, at least in part, on PLC ϵ since knock-down of PLC ϵ by more than 80% reduced PAR1 and calcium signaling by 40–50%.

A previous report [43] indicates that PAR1 coupling to InsP and calcium in astrocytes is mediated by Rho-dependent pathways. $G_{q/11}$ directly activates p63RhoGEF and $G_{12/13}$ directly activates p115RhoGEF to stimulate RhoA signaling pathways. Our data here supports the idea that the RhoA-dependent PAR1 activation of PLC observed in astrocytes [43] is mediated, in part, by PLC ϵ under the control of $G_{q/11}$ -p63RhoGEF, but not $G_{12/13}$ -p115RhoGEF, since mutant R205A specifically ablates PAR1 coupling to $G_{q/11}$ but not to $G_{12/13}$ (Figures 2 and 3). Our data does not rule out a dual role in astrocytes for $G_{q/11}$ and PLC β in this PAR1 calcium response, as is the case for PAR1 signaling in some other cells [44]. Irrespective of which $G_{q/11}$ -PLC pathway is being utilized, the key finding is that a single amino acid (R205) has been identified within the i2 loop of PAR1 that is necessary for direct PAR1 binding to $G_{q/11}$ and activation of linked InsP accumulation and calcium mobilization by one or more PLC signaling pathways.

While our studies address specific determinants within the PAR1 i2 loop that dictate G protein coupling, they do not address how PAR1 and other promiscuous GPCRs may functionally couple simultaneously to multiple G proteins in a cellular context. Agonist stimulation of PAR1 receptors in a variety of cell types clearly activates $G_{q/11}$ -linked InsP/calcium mobilization, $G_{12/13}$ -linked RhoA activation, and $G_{i/o}$ -linked inhibition of adenylyl cyclase and activation of ERK/MAPK signaling. How a single receptor is capable of coupling to all of these pathways simultaneously is not well understood. It is unlikely that a single monomeric receptor can physically couple to three or more G proteins simultaneously, especially in the case of PAR1 where the i2 and i3 loops are small relative to those of many other GPCRs. One possibility is that different subpopulations of PAR1 (perhaps in different locations?) couple simultaneously to different individual G proteins. Each G protein could couple to overlapping intracellular sites on PAR1 that include, but are not limited to the i2 loop. Our findings show that the specific residue(s) within the i2 loop are required for $G_{q/11}$ but not for $G_{i/o}$ or $G_{12/13}$ coupling, yet they do not rule out the possible involvement of the i3 loop and the C-tail for $G_{i/o}$ or $G_{12/13}$. Alternatively, PAR1 may interact with different G proteins in distinct plasma membrane compartments or subdomains. For example, PAR1 may move in and out of different microdomains in the plasma membrane such as lipid rafts and caveolae that contain different G protein partners to promote receptor/G protein complex formation and signal activation [48]. This seems to be the case for other GPCRs such as certain S1P receptors that, like PAR1, also couple to multiple G proteins [39]. Specifically, the S1P₁ receptor couples exclusively to $G_{i/o}$ depending on its compartmentalization in caveolae [49], a mechanism that also may apply to PAR1. Finally, selective PAR1/G protein coupling may also depend on the activating protease or peptide agonist. Ongoing research has supported this idea of ligand-induced functional selectivity (biased agonism), which suggests that the identity of the activating agonist plays a large role in dictating receptor/G protein coupling. In the case of PAR1, the receptor's affinity for coupling to either $G_{q/11}$ or $G_{12/13}$ shifts depending on which agonist is used to activate the receptor, thrombin or TFLLR [50].

In summary, we have identified five amino acids within the i2 loop of the PAR1 receptor that contribute to selective and efficient PAR1 coupling to $G_{q/11}$, and we show that one of these residues, Arginine 205, is essential for these interactions. Importantly, these amino acids are only important for PAR1/ $G_{q/11}$ interactions but not for PAR1 interactions with either $G_{i/o}$ or $G_{12/13}$. Our findings support a model where PAR1 relies on different intracellular regions to couple to different G proteins, and highlight previously unknown

molecular mechanisms to explain how PAR1 functionally interacts with multiple G proteins and linked signaling pathways.

Highlights for McCoy et al. “Protease-Activated Receptor 1 (PAR1) coupling to $G_{q/11}$ but not $G_{i/o}$ or $G_{12/13}$ is mediated by discrete amino acids within the receptor second intracellular loop”

- Mutating five of 21 amino acids in the PAR1-i2 loop reduce PAR1- $G_{q/11}$ signalling.
- Of these five i2 loop mutations, none affect PAR1 complex formation with $G_{i/o}$ or $G_{12/13}$, or linked downstream signalling events.
- Mutation R205A alone prevents PAR1 complex formation with G_{11} but not G_{12} or G_o .
- Mutation R205A blocks PAR1 InsP/ Ca_{++} signaling in astrocytes.
- PAR1 calcium response in astrocytes involves a $G_{q/11}$ -PLC Σ signalling pathway.

List of abbreviations

BBB	blood-brain barrier
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle medium
ECL	enhanced chemiluminescence
ERK	extracellular signal-regulated kinase
GPCR	G protein-coupled receptor
GRK2	G protein-coupled receptor kinase-2
InsP	inositol phosphate
MAPK	mitogen activated protein kinase
PAR	protease-activated receptor
PBS	phosphate-buffered saline
PLC	phospholipase C
PTX	pertussis toxin
p63RhoGEF	the 63kDa Rho guanine nucleotide exchange factor
TBS-T	Tris-buffered saline-Tween

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Figure 1.

PAR1 point mutations differentially impact receptor-activated inositol phosphate signaling. A) COS-7 cells were transfected with either vector alone (control), wild type PAR1, or the indicated PAR1 i2 loop mutant cDNA. After at least a 5 h transfection period, cells were labeled with 4 $\mu\text{Ci/mL}$ *myo*-[^3H]inositol in serum-free media overnight. The following day, cells were incubated with LiCl_2 , and then activated with 30 μM TFLLR for 30 min. To stop the InsP accumulation, cells were solubilized in formic acid, and were subsequently neutralized. After samples were subjected to anion exchange chromatography, total [^3H]InsPs were measured using liquid scintillation spectrometry. Data are presented as the percent of maximal InsP accumulation achieved by wtPAR1 (data are pooled mean results from $n=3$ separate experiments, presented mean cpm + S.E.M; each performed in triplicate). The dotted line indicates InsP accumulation of less than 50% of that stimulated with control wild type PAR1.

Figure 2. PAR1 point mutations selectively affect $G_{q/11}$ coupling without affecting $G_{i/o}$ or $G_{12/13}$ coupling. A) After a 24 h transfection with vector alone (control), wild type PAR1, or the PAR1 i2 loop mutant cDNA, COS-7 cells were lysed and harvested in sample buffer, sonicated, subjected to SDS-PAGE, and immunoblotted with anti-mCherry antibody. B) InsP accumulation was measured as described for Figure 1. Here, 10 μ M U73122 was applied to samples for 30 min prior to experimentation where indicated. C) PAR1-mediated RhoA activation was measured using a RhoA G-LISA™ Assay kit. First, vector (control), wild type PAR1, or PAR1 mutant receptor cDNA was separately transfected into COS-7 cells for 5 h before the media was replaced with serum-free media overnight. The following

day, cells were incubated with C3 toxin for 4h, where indicated. They were then activated with 30 μ M TFLLR for 30 sec before cell lysis. Experiments were performed according to the manufacturer's protocol, and the absorbances of the wells were read with a spectrophotometer at a wavelength of 490nm. D) Vector alone (control), wild type PAR1 or the PAR1 i2 loop mutants were separately transfected into COS-7 cells for at least 5 h and then serum-starved overnight in the presence or absence of 30 ng/ml PTX, as indicated. The following day, cells were stimulated with 30 μ M TFLLR for 5 min. Cells were then lysed and samples were subjected to SDS-PAGE and immunoblot. Experiments are representative results of n = 3 three separate experiments with similar results, each (for InsP and RhoA activation) performed in triplicate.

Figure 3.

Two PAR1 i2 loop mutants fail to bind directly to G_{11} whereas all mutants retain capacity to bind G_o and G_{12} . A) COS-7 cells were co-transfected with separate receptor/G protein pairs and controls (as indicated) for 24 h. Cells were then lysed, harvested, sonicated in Tris Buffer, and proteins were extracted from membranes with D β M for 3 h at 4°C. Immunoprecipitation took place overnight at 4°C with anti-FLAG resin. Recovered proteins were resolved by SDS-PAGE and proteins were immunoblotted with the indicated antibodies. *Top panel*, Western blot analysis of co-immunoprecipitated receptors and $G\alpha$ subunits. *Bottom panel*, Western blot analysis of expression levels of receptors and $G\alpha$ subunits present in cell lysates. Results are representative of three separate experiments,

each with similar results. B) COS-7 cells were transfected with either pcDNA3.1 vector alone (Con) or the indicated PAR1 i2 loop mutant cDNA. After a 24-hour transfection period, cells were labeled with *myo*-[³H]inositol for an additional 18 hr, as described in Methods. The following day, cells were activated with 30 μ M TFLLR for T = 0, 5 sec, 10 sec, 30 sec, 5 min, 10 min and 30 min after which InsP accumulation was measured as described in Methods. C) COS-7 cells were transfected with either pcDNA3.1 vector alone (Con), M1-muscarinic cholinergic receptor (M1), wild type (WT) PAR1, or the indicated PAR1 i2 loop mutant cDNA. After a 24-hour transfection period, cells were labeled with *myo*-[³H]inositol overnight and then activated with 30 μ M TFLLR for 0 or 30 min, as described in Methods. Samples were subjected to anion exchange chromatography and scintillation counting, and InsP accumulation measured as in B). Data are presented as cpm with background InsP measurements at T = 0 min subtracted out (data are pooled mean results from n=2 separate experiments, presented mean cpm $\pm$ S.E.M; each performed in triplicate).

Figure 4.

Mutant R205A disrupts PAR1-induced calcium mobilization in PAR1^{-/-} astrocytes. (A) Vector only (control), (B) wtPAR1, or the indicated (C–G) PAR1 i2 loop mutant cDNA was nucleofected into astrocytes harvested from PAR1^{-/-} mice. Two days later, the cells were loaded with Fura-2 for 30–40 min, and coverslips were transferred to a microscope stage for imaging. Imaging was performed with dual excitation at 340 nm and 380 nm wavelengths and the two resulting images were used for ratio calculations (Ex 340/Ex 380). Individual traces from 5 separate experiments for each receptor are shown superimposed on the same graph.

Figure 5. Mutant R205A disrupts PAR1-stimulated InsP signaling in PAR1^{-/-} astrocytes. Astrocytes from mice lacking the PAR1 gene (PAR1^{-/-}) were harvested and nucleofected with either plasmid vector alone (control), wild type PAR1-mCherry, or the PAR1-R205A i2 loop mutant cDNA. Two days after the nucleofection period, cells were or incubated in either serum-free media overnight (A–B) or this media containing 10 μ Ci/mL myo-[³H]inositol (C). The next day, cells were either fixed for confocal imaging (A–B) or incubated with LiCl₂ and then stimulated with 50 μ M TFLLR for 30 min (C). For the measurement of inositol phosphates (C), reactions were stopped by solubilizing astrocytes in formic acid. After samples were subjected to anion exchange chromatography, total [³H]InsPs were measured using liquid scintillation spectrometry. The accumulation of [³H]InsP is shown as counts per minute from a representative experiment (data are pooled results, mean cpm $\pm$ S.E.M, from n=2 experiments, each performed in triplicate).

Figure 6.

PLC ϵ siRNA knock-down interferes with calcium mobilization in PAR1 $^{-/-}$ astrocytes. A) Astrocytes harvested from PAR1 $^{-/-}$ mice were treated with 100 MOI PLC ϵ or random siRNA for 4 hr. Efficiency of knock-down was determined by RT-PCR three days after transduction. B–E) For calcium imaging experiments, PAR1 $^{-/-}$ astrocytes were nucleofected with wild-type (wt) PAR1 or PAR1-R205A cDNA. Two days later, the cells were treated with 100 MOI PLC ϵ or random siRNA. Following an incubation of three additional days, the astrocytes were loaded with Fura-2 for 30–40 min, and coverslips were transferred to a microscope stage for imaging. Imaging was performed with dual excitation at 340 nm and 380 nm wavelengths and the two resulting images were used for ratio

calculations (Ex 340/Ex 380). The ratio at each time point was normalized to the average ratio before stimulation with 50 μ M TFLLR. F, G) Quantification of calcium responses. The maximum responses to TFLLR (F) and ATP (G) were determined for each cell and averaged across each group.