Palmitoylation of human proteinase-activated receptor-2 differentially regulates receptor-triggered ERK1/2 activation, calcium signalling and endocytosis

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hPAR2 (human proteinase-activated receptor-2) is a member of the novel family of proteolytically activated GPCRs (G-protein-coupled receptors) termed PARs (proteinase-activated receptors). Previous pharmacological studies have found that activation of hPAR2 by mast cell tryptase can be regulated by receptor N-terminal glycosylation. In order to elucidate other post-translational modifications of hPAR2 that can regulate function, we have explored the functional role of the intracellular cysteine residue Cys361. We have demonstrated, using autoradiography, that Cys361 is the primary palmitoylation site of hPAR2. The hPAR2C361A mutant cell line displayed greater cell-surface expression compared with the wt (wild-type)-hPAR2-expressing cell line. hPAR2C361A also showed a decreased sensitivity and efficacy (intracellular calcium signalling) towards both trypsin and SLIGKV. In stark contrast, hPAR2C361A triggered greater and more prolonged ERK (extracellular-signal-regulated kinase) phosphorylation compared with that of wt-hPAR2 possibly through G, since pertussis toxin inhibited the ability of this receptor to activate ERK. Finally, flow cytometry was utilized to assess the rate and extent of receptor internalization following agonist challenge. hPAR2C361A displayed faster internalization kinetics following trypsin activation compared with wt-hPAR2, whereas SLIGKV had a negligible effect on internalization for either receptor. In conclusion, palmitoylation plays an important role in the regulation of PAR2 expression, agonist sensitivity, desensitization and internalization.

Key words: extracellular-signal-regulated kinase 1/2 (ERK1/2), G-protein-coupled receptor (GPCR), palmitoylation, proteinase-activated receptor 2 (PAR2).

INTRODUCTION

Post-translational modifications of GPCRs (G-protein-coupled receptors) can extensively regulate numerous aspects of their biology, including cell-surface expression, trafficking, folding, signalling and ligand binding. Examples of post-translational modifications known to regulate GPCR function include N-terminal glycosylation, phosphorylation and palmitoylation [1,2]. Phosphorylation of GPCRs is well known to induce receptor desensitization through the action of second-messenger-activated and GPCR kinases on serine and threonine residues located on the intracellular and C-terminal tail of the receptors [3]. Such phosphorylation results in uncoupling of the receptor from G-proteins through the involvement of β-arrestins, which bind to the phosphorylated receptors and target them towards clathrin-coated pits where the receptor undergoes endocytosis [3]. Following endocytosis, β-arrestin can target the receptor to the MAPK (mitogen-activated protein kinase) signalling pathway [4]. Thus processes that influence β-arrestin binding to a GPCR may govern which signalling pathways are triggered by the receptor.

Conflicting reports concerning PAR2 (proteinase-activated receptor-2) activation by trypside were found to be a result of N-terminal glycosylation [1,5], thus post-translational modifications of PARs can have a profound influence on receptor function and have been investigated little to date. The post-translational covalent attachment of lipid moieties is essential for many proteins. The most common lipid modification is the post-translational addition of palmitate, which also seems to have the most wide-ranging effects [6]. Palmitoylation is a thioesterification which often occurs with cysteine residues 10–14 amino acids downstream of the last transmembrane domain [7]. Using this principle we have identified a C-terminal cysteine residue (Cys361) in hPAR2 (human PAR2) as a putative palmitoylation site (Figure 1). The importance of palmitoylation in GPCRs is wide-ranging [2] and has been implicated in numerous processes, including agonist-induced internalization [8] and agonist induced down-regulation [9], but its role varies depending on the GPCR considered. Proteins can be constitutively palmitoylated or may require agonist activation for the modification to occur [10,11]. In the present study, we have investigated the role of Cys361 in various aspects of hPAR2 expression and function.

EXPERIMENTAL

Materials

Human plasma thrombin was purchased from Calbiochem/Merck. The anti-HA11 (influenza haemagglutinin) antibody was purchased from Covance. The Sam11 antibody was purchased from Zymed/Invitrogen. FBS (fetal bovine serum), Dulbecco’s α-MEM (α-modified essential medium) with ribonucleosides and deoxyribonucleosides, non-enzymic cell-dissociation solution, genetin, penicillin, streptomycin, PBS (without calcium and}

Abbreviations used: α-MEM, α-modified essential medium; AP-2, activating protein-2; ECL, enhanced chemiluminescence; ERK1/2, extracellular-signal-regulated kinase 1/2; FBS, fetal bovine serum; fluo-3 AM; fluo-3 acecytoxymethyl ester; GPCR, G-protein-coupled receptor; HA11, influenza haemagglutinin; hPAR2, human proteinase-activated receptor-2; MAPK, mitogen-activated protein kinase; NT, no treatment; OTR, oxytocin receptor; PAR, proteinase-activated receptor; PLC, phospholipase C; PTX, pertussis toxin; PYK2, proline-rich tyrosine kinase 2; RT, room temperature; wt, wild-type.

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magnesium), pcDNA3.1(+) and fluo-3 AM (fluo-3 acetoxyethyl ester) were from Invitrogen. All oligonucleotides were synthesized by the Peptide Synthesis Facility, University of Calgary, Alberta, Canada, or were purchased from Peptides International. The μMACSTM HA11 Epitope Tag Protein Isolation kit was supplied by Miltenyi. The Hybond C PVDF membrane, Hyperfilm™ X-ray film, the peroxidase-conjugated goat anti-mouse antibody, Amplify™ and ECL (enhanced chemiluminescence) kit were purchased from Amersham Biosciences/GE Healthcare. All other chemicals and reagents were purchased from Sigma unless otherwise stated.

Expression vectors

The wt (wild-type)-hPAR2 cDNA possessing a C-terminal HA11 epitope (YPYDVPDYA) used in the present study has been described in detail previously [1]. Human PAR2C361A was generated using the QuickChange® site-directed mutagenesis kit (Stratagene), according to the manufacturer’s instructions. The engineered mutation in PAR2 was then confirmed by automated DNA sequencing (MWG).

Cell culture and transfection

The Pro5 CHO (Chinese-hamster ovary) cell line permanently expressing either wt-hPAR2 or mutant hPAR2 was propagated in selective growth medium (α-MEM containing 10% FBS, 100 units/ml penicillin, 100 ng/ml streptomycin, 250 ng/ml amphotericin B and 800 μg/ml genetin) in 5% CO₂ at 37°C. All cell lines were harvested using non-enzymatic cell-dissociation solution.

Permanent receptor-expressing cell lines were generated by transfection using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer’s protocol. Transfected Pro5 cells were propagated in selective growth medium for 2 weeks to allow selection. Permanently expressing cell lines were selected by single cell cloning and were assessed for receptor expression by flow cytometry.

Determination of cell-surface expression of wt-hPAR2 and hPAR2C361A

It has been found previously that in the cell lines used ‘receptor expression (is) sensitive to confluence’ [1]. Therefore flow cytometry was used to assess receptor expression. Cells were grown to different levels of confluence and then labelled with the Sam11 mouse anti-PAR2 antibody and FITC-conjugated anti-

mouse antibody before analysing with flow cytometry to assess and match receptor expression.

Localization of receptors by confocal imaging

Confocal microscopy was used to determine the location of wt-PAR2 and PAR2C361A within the cells. Sam11 indicated cell-surface expression and HA11 indicated total cell expression. For Sam11 staining, cells grown on coverslips were incubated with the Sam11 anti-PAR2 antibody (1 μg/ml) at RT (room temperature; 21°C) for 60 min before incubation with the FITC-conjugated anti-mouse antibody (1 μg/ml). For HA11 staining, coverslips were pre-incubated in PBS (containing 1% BSA and 3% rabbit serum) for 15 min prior to the addition of the mouse monoclonal anti-HA11 antibody (1 μg/ml) and were incubated at RT for 60 min. Coverslips were then incubated with the FITC-conjugated anti-mouse antibody (1 μg/ml). The slides were then analysed on a confocal microscope using an argon laser exciting at 488 nm and detecting fluorescence emissions above 500 nm. Each experiment consisted of two pictures from each of three coverslip repeats for each treatment and a final consensus photograph being chosen.

[3H]Palmitate labelling and PAR2 immunoprecipitation

The constitutive palmitoylation of PAR2 was investigated by detecting [3H]palmitate labelling of the receptor. wt-hPAR2- and hPAR2C361A-expressing Pro5 cell lines were seeded in 100-mm dishes, pre-incubated overnight in serum-free medium and labelled with 1 μCi of [3H]palmitate for 4 h at 37°C. After labelling, PAR2 protein from the cell lines was isolated by immunoprecipitation using the μMACSTM HA11 Epitope Tag Protein Isolation kit, according to the manufacturer’s protocol.

Proteins were then resolved by SDS/PAGE under non-reducing conditions and then fixed for 30 min in propan-2-ol/water/acetic acid (25:62:10, by vol.). Gels were then treated with Amplify™ for 15 min at RT before drying under vacuum at 80°C. The dried gel was then exposed to Hyperfilm™ X-ray film at −80°C for 24 h, before developing.

In order to confirm equal loading of radiolabelled PAR2 protein, immunoblots were performed with half of the protein sample used in the palmitate experiments. Briefly, immunoprecipitated samples were separated by SDS/PAGE (10% gel) before transfer onto to a Hybond C PVDF membrane. The membrane was blocked with 5% non-fat milk powder in PBS/0.1% Tween 20 for 1 h before incubation overnight at 4°C with the mouse monoclonal anti-HA11 antibody (1:1000 dilution in PBS/0.1% Tween 20 containing 2% non-fat milk powder) and then incubated with peroxidase-conjugated goat anti-mouse antibody (1:1000 dilution) for 1 h. The epitope-tagged hPAR2 was then visualized using ECL (ECL2), captured on a UVP Epi Chem II Darkroom with a fluorescence camera and analysed using Labworks v4.5 software.

Calcium signalling assay

Calcium signalling was performed as described previously [12]. Cells were harvested non-enzymically and incubated in 1 ml of α-MEM containing 0.25 mM sulphinpyrazone and 22 μM fluo-3 AM for 25 min at RT on an orbital mixer. Cells were then washed and resuspended in calcium assay buffer (150 mM NaCl, 3 mM KCl, 1.5 mM CaCl₂, 10 mM glucose, 20 mM Hepes and 0.25 mM sulphinpyrazone, pH 7.4). Fluorescence measurements were performed on a Photon Technology International Fluorospectrophotometer, with an excitation wavelength of 480 nm and emission recorded at...
530 nm. The signal produced by the addition of a test agonist was measured as a percentage of the fluorescence peak height, yielded by the addition of 2 μM A23187, a calcium ionophore.

**ERK1/2 (extracellular-signal-regulated kinase 1/2) phosphorylation and immunoblots**

Cells were grown in six-well plates to a confluence of 40–50% in normal medium. The medium was then replaced with serum-free medium and cells were incubated overnight. Cells were then treated with agonist for a specified time (0, 5, 10, 30 and 60 min) before halting treatment and harvesting the cells in Laemlli’s sample buffer (30% glycerol, 140 mM Tris/HCl, pH 6.8, 5% SDS, 7 mM Bromophenol Blue, 5 mM EDTA and 1/20 2-mercaptoethanol). Samples were then incubated at 100°C for 5 min prior to analysis by Western blotting (as described above) using the anti-(p44/42 MAPK) (1:1000 dilution) (Cell Signaling Technology).

**Receptor internalization assay**

Semi-confluent cells (approx. 40% confluence) were harvested and treated with trypsin (100 nM) or the synthetic peptide SLIGKV (100 μM), which corresponds to the tethered ligand exposed by trypsin cleavage of PAR2, for 0–30 min at RT before being placed on ice. Cells were then washed and centrifuged. Pelleted cells were resuspended in ice-cold PBS and placed on ice for 10 min before incubation with the Sam11 mouse anti-PAR2 monoclonal antibody (3 μg/ml) for 1 h on ice, followed by incubation with FITC-conjugated anti-mouse antibody (10 μg/ml) for a further 45 min. Cells were then analysed for PAR2-cell-surface expression by flow cytometry (Becton Dickinson).

**Statistical analysis**

Graphs were produced using GraphPad Prism. For a two-dataset comparison of group means, a two-tailed test was used. When this was to compare two different treatments on the same cell line or comparison of two different time courses or concentration ranges that of wt-PAR2.

**RESULTS**

**Expression and palmitoylated state of wt-PAR2 and PAR2C361A**

In order to study the potential role of palmitoylation in regulating PAR2 function, Pro5 cells were stably transfected with HA11-tagged wt-hPAR2 or hPAR2C361A, where the putative palmitoylation site (Cys61) had been replaced with an alanine residue. Receptor-expressing cell lines were established and used at confluences which resulted in similar receptor expression, as determined by flow cytometry (Figure 2). These were subsequently employed for further studies.

Confocal imaging was used to identify the cellular localization of receptor expression (Figure 3). When stained with the Sam11 anti-PAR2 antibody, empty-vector-transfected cells (Figure 3A) showed no staining, whereas Pro5 cells expressing wt-hPAR2 (Figure 3B) and hPAR2C361A (Figure 3C) showed staining at the cell membrane. However, low levels of fluorescence were observed using this method and a high photomultiplier gain was required to observe any signal. Cells were therefore permeabilized and stained with the anti-HA11 antibody. wt-hPAR2 (Figure 3E) displayed distinct, uniform and robust immunostaining around the cell membrane. Some punctate cytoplasmic staining was also observed. No staining was observed in the empty-vector-transfected cells (Figure 3D). The staining pattern observed for hPAR2C361A (Figure 3F) was strikingly different to that of wt-hPAR2 (Figure 3E). The ring staining for hPAR2C361A was not uniform and distinct pockets of intense staining were observed at the cell surface. In addition, considerable punctate staining was observed in the cytoplasmic region of the cells (Figure 3F). However, the staining for hPAR2C361A appeared brighter than that of wt-PAR2.

The palmitoylation state of wt-hPAR2 and hPAR2C361A was then determined by metabolic labelling of the cells with [3H]palmitoyl-CoA, followed by immunoprecipitation of the receptors. Figure 4(A) shows that detectable levels of [3H]were incorporated into wt-PAR2. Two broad smears were observed, one between ~50 and 75 kDa and a second from ~25 to 30 kDa. In contrast, PAR2C361A displayed an undetectable level of [3H]incorporation. Western blot analysis of the same samples that were employed for the palmitoylation gel demonstrated that approximately equal amounts of PAR2 protein were present in the wt-PAR2 and PAR2C361A samples (Figure 4B).

**Role of palmitoylation in regulating intracellular calcium signalling**

To assess the importance of palmitoylation in regulating hPAR2 coupling to calcium, we compared the ability of wt-hPAR2 and hPAR2C361A to trigger increases in intracellular calcium in response to the hPAR2 agonists trypsin and SLIGKV. Trypsin and SLIGKV concentration–effect curves for hPAR2 and hPAR2C361A cell lines with matched expression (Figure 2) were constructed (Figure 5). For wt-hPAR2, both trypsin and SLIGKV stimulated robust and similar maximal responses (~60% of A233187) at 316 nM and 316 μM respectively (Figure 5). The EC50 for trypsin and SLIGKV in activating wt-PAR2 was 11.44 nM and 15.69 μM respectively. In contrast with wt-hPAR2, trypsin and SLIGKV displayed significantly reduced efficacy towards hPAR2C361A (P = 0.0003 and P = 0.0058 respectively). The
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Figure 3 Confocal images of wt-hPAR2 and hPAR2C361A localization within the cell

Cells were incubated with the primary Sam11 anti-PAR2 antibody and the secondary FITC-conjugated anti-mouse antibody. Coverslips were then mounted and analysed by confocal microscopy. (A) pcDNA3.1 empty vector, (B) wt-hPAR2 and (C) hPAR2C361A. Fixed and permeabilized cells were incubated with primary anti-HA11 antibody and secondary FITC-conjugated anti-mouse antibody before being analysed by confocal microscopy. (D) pcDNA3.1 empty vector, (E) wt-hPAR2 and (F) hPAR2C361A. The presence of PAR2 at the plasma membrane is indicated with arrowheads. PAR2 contained internally is indicated with arrows. Images shown are representative of three experiments.

Figure 4 [3H]Palmitate incorporation into wt-hPAR2 and hPAR2C361A

HA11-epitope-immunoprecipitated protein preparations from empty vector, wt-hPAR2 and hPAR2C361A cells each labelled with 1 mCi of [3H]palmitate for 4 h were separated by SDS/PAGE on a 10% gel. The gel was dried under vacuum and was exposed to Hyperfilm™ X-ray film at −80°C for 6 weeks (A). A portion of the same samples were analysed by Western blotting using the anti-HA11 antibody and a 20 min exposure time to assess receptor loading (B). The blots shown are representative of three experiments. Molecular-mass markers are shown on the left-hand side.

maximal obtainable responses to both trypsin and SLIGKV were only ~50% and ~30% respectively of that observed for wt-hPAR2 (Figure 5). The hPAR2C361A EC50 values for trypsin and SLIGKV were 40.76 nM and 103.45 μM respectively, displaying a 4- and 6-fold increase compared with that obtained for wt-hPAR2.

Inhibition of trypsin-mediated calcium signalling by PTX (pertussis toxin)

PTX was used to ascertain what proportion of the total Ins(1,4,5)P3-induced calcium flux is due to signalling through Gi in wt-hPAR2 and the palmitoylation-deficient mutant hPAR2C361A. PTX dose-dependently inhibited wt-hPAR2- and hPAR2C361A-mediated calcium signalling to trypsin. wt-hPAR2 generated a maximum calcium signal of ~70% of A23187, decreasing to ~50% of A23187 at the maximum PTX concentration of 316 ng/ml (Figure 6). In contrast, hPAR2C361A produced a maximum calcium signal of ~30% of A23187, reducing to <10% of A23187 at the maximum PTX concentration of 316 ng/ml. As such, both curves were reduced by ~20% of their A23187 signal (Figure 6).

Role of palmitoylation in regulating ERK1/2 phosphorylation

Having established that the palmitoylation-deficient hPAR2C361A mutant coupled less efficiently to calcium, we investigated whether receptor palmitoylation possibly regulated hPAR2 signalling to ERK1/2 (p44/42 MAPK). Significant differences in the pattern of the ERK1/2 stimulation were observed for hPAR2C361A compared with wt-hPAR2 (P < 0.001; Figure 7A). SLIGKV stimulated a significantly
PAR2 is regulated by palmitoylation

Figure 5 Agonist concentration–effect curves for wt-hPAR2 and hPAR2C361A

Fluo-3 AM-loaded Pro5 cells stably expressing wt-hPAR2 and hPAR2C361A were stimulated with increasing concentrations of trypsin or SLIGKV, and increases in intracellular calcium concentrations were measured. Results are expressed as a percentage of the maximum attainable response (response to the calcium ionophore A23187), and are means ± S.E.M. for four separate experiments each performed in duplicate.

Figure 6 Inhibition of trypsin-mediated calcium release in wt-hPAR2 and hPAR2C361A

Changes in intracellular calcium levels were measured using a fluorospectrometer in fluo-3 AM-loaded wt-hPAR2 and hPAR2C361A incubated with different concentrations of PTX (for 18 h) prior to the addition of 100 nM trypsin. Values shown are a percentage of the maximum attainable response (response to A23187) and are means ± S.E.M.

greater degree of ERK1/2 phosphorylation in hPAR2C361A cells at the 5 and 10 min time points, and the ERK1/2 activation levels remaining significantly elevated up to the 60 min time point tested. Similarly, trypsin was also found to stimulate a significantly greater degree of ERK1/2 phosphorylation through hPAR2C361A compared with wt-hPAR2 (P < 0.001; Figure 7B). Trypsin stimulated a significantly greater degree of ERK1/2 phosphorylation in hPAR2C361A cells at the 5, 10 and 20 min time points, and a more sustained ERK1/2 activation level remaining significantly elevated up to the 60 min time point tested. All phospho-ERK1/2 blots were stripped of antibodies and re-probed with anti-(total ERK1/2) antibodies. Phospho-ERK1/2 levels were then normalized against total ERK1/2 and expressed as a fold change of NT (no treatment).

Figure 7 Agonist-stimulated MAPK phosphorylation

Representative phospho-ERK1/2 and total ERK1/2 immunoblots (p42/44) as determined by Western blot analysis for post-SLIGKV treatment (A) and post-trypsin treatment (B). The graphs below the immunoblots show the densitometric values for the bands from the Western blot analysis of phospho-ERK1/2 (p44/42). Normalized values are expressed as a fold change over NT. Results are means ± S.E.M. for three separate experiments.

Inhibition of MAPK signalling by PTX

To ascertain what proportion of the ERK signalling is due to signalling through Gi, and to determine whether the lack of palmitoylation altered this signalling, wt-hPAR2, hPAR2C361A and empty vector control cells were pre-incubated with PTX before treating with PAR2 agonists (trypsin and SLIGKV).

Trypsin and SLIGKV evoked negligible changes in ERK1/2 signalling compared with NT in the empty vector cells (results not shown). Treatment with PTX caused no significant change in ERK1/2-agonist-induced signalling. For wt-hPAR2 (Figure 8A), SLIGKV and trypsin caused a 2- and 1.5-fold increase in ERK1/2 signal over NT respectively. Following pre-treatment with PTX there appeared to be a slight decrease in ERK1/2 signalling with all treatments, although no significant difference was observed. SLIGKV and trypsin evoked a 2- and 3-fold increase in ERK1/2 signalling over NT respectively in hPAR2C361A-expressing cells (Figure 8B). When pre-treated with PTX, ERK1/2 signalling in hPAR2C361A-expressing cells decreased a small amount following NT and trypsin treatment in comparison with samples not pre-treated with PTX. However, a large fall in ERK1/2 signalling was observed in the PTX-pre-incubated SLIGKV-treated samples compared with those not pre-incubated with PTX, although the changes were not statistically significant.
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**Agonist-triggered internalization of PAR2**

Flow cytometry was employed to determine the agonist-induced internalization of PAR2 using the monoclonal Sam11 antibody, which recognizes a sequence of the N-terminal domain that is located C-terminal to the tethered-ligand [14] (Figure 9). Thus receptors that have been activated by trypsin can still be readily detected by this antibody [14]. As shown in Figure 9, trypsin (100 nM) promoted rapid and significant (~50%) loss of cell-surface wt-PAR2 by 10 min post-agonist challenge. At the 60 min time point, ~42% of wt-PAR2 was still detectable at the cell surface. Curiously, SLIGKV (100 μM) only stimulated a modest loss of cell-surface receptor at 30 and 60 min post-agonist challenge. In contrast with wt-hPAR2, trypsin stimulated a significantly greater loss of hPAR2C361A from the cell surface at all of the time points tested, with over 81% ± 1.3% lost from the cell surface by 10 min post-agonist challenge ($P = 0.016$). Maximal loss of cell-surface receptor was observed by 20 min (95 ± 2.6%), which thereafter remained unchanged up to the 60 min time point. As with wt-hPAR2, we also observed only a minor degree of receptor internalization with hPAR2C361A following treatment with SLIGKV, with maximal internalization observed at 60 min (16 ± 2%).

**DISCUSSION**

The results described in the present study provide the first direct biochemical and molecular evidence that hPAR2 is palmitoylated and that Cys361 is likely to be the primary site of palmitoylation. In addition, evidence is presented demonstrating that palmitoylation of hPAR2 is a dynamic process that is influenced by agonist activation. Furthermore, palmitoylation of hPAR2 was shown to regulate constitutive receptor expression, agonist-triggered internalization and, more importantly, inversely regulate receptor signalling to two major signalling pathways, namely calcium and MAPK.

The hPAR2C361A cell line displayed greater receptor expression than the wt-hPAR2 cell line. Removal of palmitoylation often results in a loss of cell-surface expression as seen with the V2 vasopressin receptor and CCR5 (CC chemokine receptor 5) [15–17]. To our knowledge, the present study is the first to find an increase in receptor expression resulting from the mutagenic removal of a palmitoylated cysteine residue. Possible reasons for increased receptor expression are discussed below.

Confocal microscopy was employed next to compare the cellular distribution of wt-hPAR2 and hPAR2C361A. Confocal imaging shows that PAR2 is located at the plasma membrane, along with some internal localization (which is greater in hPAR2C361A); this is presumably, at least in part, PAR2 contained in Golgi stores, which is confirmatory of previous confocal imaging of PAR2 [18–20].

Immunoprecipitation of wt-hPAR2 and hPAR2C361A was carried out using the HA11 epitope tag, and Western blotting was carried out using the anti-HA11 antibody, as Sam11 does not work well in Western blot analysis. As half of the sample was used to detect palmitoylation, immunoprecipitation was therefore essential to remove unwanted (non-HA11 tagged) proteins. The banding pattern observed is in keeping with the previous immunoblotting of hPAR2 [1] and the C361A appears...
to have no perceptible effect on the receptor molecular mass compared with wt-hPAR2. Despite flow cytometry results showing higher hPAR,C361A expression than wt-hPAR2, three times more cells were required to achieve matching quantities of receptor. However, if hPAR2 is palmitoylated at Cys\(^{361}\), the C361A mutation will result in a reduced C-terminal association with the cell membrane and potentially leave the C-terminus available to be more easily cleaved by endogenous proteases, thus removing the HA11 tag. Alternatively, the epitope may be obscured by the C-terminus becoming associated with different receptor domains or sterically by an accessory protein binding to a nearby region on the C-terminus [21,22].

Using direct biochemical labelling, we provide compelling evidence that hPAR2 is palmitoylated. There is evidence that GPCRs can be palmitoylated in regions other than the C-terminal tail [23]. However, since no \(^{3}H\)palmitate signal is evident in hPAR,C361A, it seems likely that this is the sole palmitoylation site for hPAR2.

PAR2 palmitoylation has a significant effect on receptor-mediated calcium signalling. The decreased signal and agonist sensitivity observed for the hPAR,C361A mutant may be due to the revealing of phosphorylation sites within the C-terminus, which may normally be obscured by association with the membrane when palmitoylation is present. This is similar to the β-adrenergic receptor, where substitution of the palmitoylated cysteine residue appears to allow agonist-independent phosphorylation of receptor PKA (protein kinase A) and GRK (G-protein-coupled-receptor kinase) sites which would normally only phosphorylate upon activation [24]. In other GPCRs, desensitization/internalization can be driven by phosphorylation-dependent interactions with β-arrestin [24–26]. Mutagenic replacement of the hPAR2 palmitoylation site may alternatively cause a conformational change in the receptor, leaving the tethered ligand and activating peptide incapable of correctly binding to the ligand-binding site. Although this has not been seen previously in the mutagenic replacement of palmitoylation, removal and mutagenesis of areas of the C-terminal tail in some GPCRs have been shown to affect receptor folding [27,28]. The reduction in receptor-mediated calcium signalling may be as a result of altered G-protein coupling. The C-terminal tail of many GPCRs is associated with G-protein coupling and, as such, removal of the palmitoylation site affects G-protein coupling in many GPCRs [24,26,29,30]. In some, this is most likely to be because of increased phosphorylation [24–26,31], but some mutagenically depalmitoylated GPCRs have decreased G-protein coupling without displaying increased phosphorylation [29,32], suggesting another mechanism.

Experiments assessing MAPK activation revealed that palmitoylation of PAR2 inversely regulates MAPK activation. This increased ERK1/2 signalling seen with the non-palmitoylated mutant may be due to increased β-arrestin association. It is well known that phosphorylation of the C-terminus of PAR2 results in an increased affinity for β-arrestin binding which, in turn, uncouples the receptor from its G-protein by a steric mechanism [18–20]. Additionally, PAR2 expressed alongside a β-arrestin mutant incapable of receptor binding shows a diminished ability to activate ERK1/2 [18]. Studies on a number of GPCRs have now demonstrated that β-arrestins can mediate a number of signalling pathways independent of G-proteins [33,34]. PAR2 activation has also been shown to promote β-arrestin actions independent of G-proteins by direct inhibition of G\(_{\text{a11}}\), as well as receptor coupling to G\(_{\text{a11}}\) [35]. There is additional evidence to suggest that residues in the C-terminus of PAR2, define the specificity of β-arrestin binding and the duration of ERK1/2 association with β-arrestin [18,36,37]. This combined with β-arrestin 1 and 2 association with ERK1/2 activation in the early and intermediate phases [38] supports the hypothesis that ERK1/2 signalling in hPAR,C361A may be enhanced due to an increase in β-arrestin association. This increased β-arrestin association may also explain the increased receptor expression seen in hPAR,C361A compared with wt-hPAR2, as studies have shown a role for β-arrestins in trafficking of PAR2 from the Golgi [35,38]. Further studies to investigate the role of β-arrestins in hPAR,C361A signalling, expression and internalization are required. Alternatively, palmitoylation is linked with the targeting of receptors and signalling molecules alike to specific membrane microdomains such as lipid rafts [39,40]. It has been shown that G\(_{\text{aq}}\) interacts with caveolin, while as G\(_{\text{ia}}\) does not, suggesting they may therefore be targeted to different lipid regions [41]. OTR (oxytocin receptor) is known to couple to both G\(_{\text{q}}\) and G\(_{\text{i}}\), but differentially activates one or the other depending on its membrane localization [42]. When located within lipid rafts, OTR activation results in cell growth through a G\(_{\text{q}}\)-mediated pathway; when outside lipid rafts, it results in G\(_{\text{i}}\)-mediated inhibition of cell growth. This effect on cell growth has been shown to be as a result of a different temporal pattern of EGFR (epidermal growth factor receptor) and ERK1/2 phosphorylation, and has been shown to be more persistent when receptors are located outside of lipid raft microdomains [42]. As such, palmitoylation in hPAR2 may target the receptor to specific lipid raft microdomains, removal of which results in movement outside of these lipid rafts resulting in greater G\(_{\text{i}}\) association and prolonged ERK1/2 signalling.

Palmitoylation has been shown to affect the ability of a GPCR to couple to its respective G\(_{\text{i}}\) subunit. For some GPCRs, palmitoylation has been shown to selectively effect coupling to specific G\(_{\text{i}}\) subunits [29,32], removing coupling to one or more subunits while maintaining coupling with another. PTX, an inhibitor of signalling through G\(_{\text{i}}\), was used to understand further the effect of palmitoylation on PAR2 G-protein-coupled calcium signalling. Interestingly, calcium signalling of wt-hPAR2 and hPAR,C361A was reduced by a similar magnitude; however, in the mutant receptor, this reduction almost completely ablated the trypsin-evoked calcium response, suggesting that signalling via hPAR,C361A is due to coupling with G\(_{\text{i}}\). Although previous GPCRs have shown selective uncoupling of G\(_{\text{i}}\) subunits [29,32], these have preferentially lost G\(_{\text{i6}}\) and maintained G\(_{\text{i1}}\) coupling. This was suggested by Okomoto et al. [40] as being due to G\(_{\text{i6}}\) requiring a higher level of palmitoylation. However, if palmitoylation of hPAR2 targets the receptor to G\(_{\text{i6}}\)-containing lipid rafts, as with the OTR [42], and removal of palmitoylation results in the receptor located outside of the raft, then association with G\(_{\text{i6}}\) would be disrupted. A recent study has suggested that PAR2, unlike PAR1, does not signal through G\(_{\text{i6}}\) [43]; however, in our cell system, it appears that in order to provoke calcium signalling hPAR2,C361A couples to G\(_{\text{i6}}\). As reported by DeFea et al. [18], we have shown that hPAR2 activates ERK1/2 through a PTX-insensitive pathway, thus having little or no involvement by G\(_{\text{i6}}\). In contrast, the non-palmitoylated hPAR2,C361A displayed reduced ERK phosphorylation post-SLIGKV activation, suggesting a G\(_{\text{i1}}\)-mediated response. Numerous studies have shown ERK activation to be independent of G-protein signalling, and PAR2-induced ERK signalling has been shown to be mediated by β-arrestin association [35,38]. It maybe that the increased association with G\(_{\text{i1}}\) results in a shift in the preferred pathway for ERK1/2 activation. The signalling pathway responsible for the activation of ERK in PAR2 has been shown previously to ‘switch’ in the case of PAR\(_{\text{δ}}\)ST363/366A, a phosphorylation-deficient mutant, which activated ERK1/2 via a PYK2 (proline-rich tyrosine kinase 2)-mediated pathway [18]. It was hypothesized that the interaction with the PYK2 pathway was a result of the prolonged calcium mobilization observed in...
this mutant. As stated earlier, calcium mobilization following hPAR2C361A activation takes longer to reach a signalling plateau, thus it maybe that the Gq-dependent activation of ERK1/2 activation observed is PYK2-mediated. In addition to Gq and Gs, PAR2 is also thought to signal through the Gi12/13 and Rho pathways [43]. Although we have not investigated these pathways in the present study, it would be interesting to determine the effect of PAR2 palmitoylation status on these pathways.

The rate and extent of internalization in response to trypsin is increased in the palmitoylated mutant compared with wt-hPAR2. However, neither mutant nor wt-PAR2 showed internalization following treatment with SLIGKV. This is in contrast with what has been reported previously for PAR2 following SLIGKV treatment [18–20]. Internalization, rather than receptor degradation, of PAR2 was confirmed by permeabilizing cells and using the endocytic and PKC (protein kinase C) inhibitors concanavalin A and hyperosmolar glucose, as well as fixing the membrane (results not shown). Hypertonic sucrose is known to prevent the recruitment of clathrin and interferes with normal coated-pit formation and endocytosis [44,45], and concanavalin A is known to prevent the formation of coated pits [46,47]. As such we have also demonstrated that hPAR2 internalizes by clathrin-coated pits, and hPAR2c361A internalizes by the same mechanism albeit more rapidly. Although the present study is the first to report a differential effect of receptor cell-surface expression and post-agonist endocytosis with trypsin and SLIGKV, the internalization seen post-SLIGKV treatment in previous publications may be due to overexpression of β-arrestin in the cell lines used. A previous study has shown overexpression of β-arrestin to result in its constitutive association with cellular components [35].

Despite increased expression of hPAR2C361A compared with wt-PAR2 in the resting state, with the addition of agonists the endocytosis of the mutant receptor appears to be increased over wt-PAR2. This enhanced post-activation endocytosis may be for a number of different reasons. If, as discussed previously, hPAR2C361A is constitutively phosphorylated then β-arrestin may associate with the receptor C-terminus. For the conformational change to occur in β-arrestin that allows high-affinity binding of AP-2 (activating protein-2) and clathrin, resulting in endocytosis, it must be bound to a receptor that is both phosphorylated and activated [48]. Since β-arrestin is already associated with the receptor upon activation, endocytosis can occur with fewer binding steps. If hPAR2 functions similarly to OTR, then following activation the receptor depalmitoylates and needs to translocate from the caveola domain before being endocytosed [42]. The removal of hPAR2 palmitoylation would mean the receptor was already located outside of the caveola domain, allowing clathrin binding and endocytosis without the need for receptor translocation. The increase in the amount of receptor endocytosed post-activation may occur as a result of uncoupling from Gs; PLC (phospholipase C) activity has been shown previously to inhibit endocytosis via clathrin-coated pits [49]. Since hPAR2C361A has reduced/abolished coupling to Gs, PLC activity would be significantly reduced/abolished, thus allowing increased endocytosis via clathrin-coated pits in the absence of inhibition from PLC.

Upon activation the receptor must become depalmitoylated and phosphorylated before β-arrestin binding. In order to mediate receptor endocytosis, β-arrestin must undergo a conformational change which increases the binding affinity of clathrin and AP-2 [48]. For this conformational change to occur, the receptor must be both in an activated confirmation and phosphorylated [48]. Additionally, receptor phosphorylation has been shown previously to be the rate-limiting step for β-arrestin association [50]. As stated above, it may be that the removal of palmitoylation results in the receptor being phosphorylated due the increased availability of the C-terminal phosphorylation sites, thus allowing β-arrestin binding, but endocytosis does not occur until the receptor is activated. Since β-arrestin is already associated with the receptor, endocytosis can occur immediately without initial β-arrestin association. It would be interesting to investigate this by further experimentation.

In conclusion, the present study is the first to investigate the palmitoylation status of hPAR2 and its effect on receptor function. We have demonstrated that hPAR2 is palmitoylated predominantly, if not entirely, on CysC361. We have demonstrated further the multifaceted importance of palmitoylation on receptor expression, agonist sensitivity, densensitization and internalization.

**AUTHOR CONTRIBUTION**

Andrew Botham performed most of the experimental work; Xiaodan Guo performed some of the palmitoylation blots; Yu Pei Xiao prepared some of the Figures and performed the statistics; Alyn Morice and Steven Compton designed and supervised the project; Laura Sadovsky performed some of the Western blots and prepared the paper.

**FUNDING**

This study was supported by a British Heart Foundation Ph.D. Studentship [grant number FS/03/078/15918].

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Received 25 November 2010/24 May 2011; accepted 31 May 2011
Published as BJ Immediate Publication 31 May 2011, doi:10.1042/BJ20101958

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