Palmitoylation of Human Proteinase-Activated Receptor-2 Differentially Regulates Receptor Triggered ERK1/2 Activation, Calcium Signalling, and Endocytosis.

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Running Head: PAR_2 is regulated by Palmitoylation
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$hPAR_2$, is a member of the novel family of proteolytically-activated G-protein coupled receptors (GPCR) termed Proteinase-Activated Receptors (PAR). Previous pharmacological studies have found that activation of $hPAR_2$ by mast cell tryptase, can be regulated by receptor *N*-terminal glycosylation. In order to elucidate other post-translational modifications of $hPAR_2$ that can regulate function, we have explored the functional role of the intracellular cysteine, C361. We demonstrated, using autoradiography, that C361 is the primary palmitoylation site of $hPAR_2$. The $hPAR_2$C361A mutant cell line displayed greater cell surface expression compared to the wt-$hPAR_2$ expressing cell line. The $hPAR_2$C361A also showed a decreased sensitivity and efficacy (intracellular calcium signalling) towards both trypsin and SLIGKV-NH_2. In stark contrast $hPAR_2$C361A triggered greater and more prolonged ERK phosphorylation compared to that of wt-$hPAR_2$ possibly through Gi, since pertussis toxin inhibited this receptors ability to activate ERK. Finally, flow cytometry was utilised to assess the rate, and extent of receptor internalisation following agonist challenge. The $hPAR_2$C361A receptor displayed faster internalisation kinetics following trypsin activation, compared to wt-$hPAR_2$, whilst SLIGKV-NH_2 had negligible effect on internalisation for either receptor. Palmitoylation plays an important role in the regulation of PAR_2 receptor expression, agonist sensitivity, desensitisation and internalisation.

Keywords: ERK1/2, GPCR, Palmitoylation, PAR_2

INTRODUCTION

Post-translational modifications of GPCRs 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*-linked glycosylation, phosphorylation and palmitoylation [1-2]. Phosphorylation of GPCRs is well known to induce receptor desensitisation through the action of second messenger-activated and GPCR kinases on serine and threonine residues located on the intracellular and carboxyl 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 clatherin coated pits where the receptor undergoes endocytosis [3]. Following endocytosis, β-arrestin can target the receptor to
the mitogen-activated protein kinases (MAPK) 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 PAR_2 activation by tryptase were found to be a result of N-linked glycosylation [1, 5], thus post-translational modifications of PARs can have a profound influence on receptor function and have to date been little investigated. The post-translational covalent attachment of lipid moieties is essential for many proteins. The most common lipid modification is the post-translational addition of palmitic acid, which also seems to have the most wide-ranging effects [6]. Palmitoylation is a thioesterification which often occurs with cyteine

Abbreviations: α-MEM, Dulbecco's α-modified essential medium; ANOVA, analysis of variance; AP, activating protein; CHO, Chinese hamster ovary; ERK1/2, extracellular signal-regulated kinases; FCS, foetal calf serum; GPCR, G-Protein coupled receptor; HA11, influenza haemagglutinin; $hPAR_2$, human proteinase activated receptor; MAPK, mitogen activated protein kinase; PTX, pertussis toxin; RT, room temperature.

residues 10-14 amino acids downstream of the last transmembrane domain [7]. Using this principal we have identified a C-terminal cysteine residue in $hPAR_2$ (C361) 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 internalisation [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 this study we investigate the role of C361 in various aspects of $hPAR_2$ expression and function.

EXPERIMENTAL

Materials.

Human plasma thrombin was purchased from Calbiochem, Merck Chemicals Ltd, (Nottingham, UK). Anti-HA11 antibody was purchased from Covance (Harrogate, UK). Sam11 antibody was purchased from Zymed (Invitrogen, Paisley, UK). Foetal calf serum (FCS), Dulbecco's α-modified essential medium (α-MEM, with ribonucleosides and deoxyribonucleosides), non-enzymic cell-dissociation solution, geneticin, penicillin, streptomycin, PBS (without calcium and magnesium) pcDNA 3.1(+) and fluo-3 acetoxymethyl ester (fluo-3 AM) were from Invitrogen (Paisley, UK). All oligonucleotides were synthesized by the peptide synthesis facility, University of Calgary (Alberta, Canada) or purchased from peptides international (Kentucky, USA). µMACS™ HA11 Epitope Tag Protein Isolation Kit were supplied by Miltenyi Biotech (Bergisch, Germany). Hybond C PVDF membrane, hyperfilm x-ray film, peroxidase conjugated goat anti-mouse IgG, Amplify™ and ECL was purchased from Amersham Biosciences (Buckinghamshire, U.K.). All other chemicals and reagents were purchased from Sigma (Dorset, UK.) unless otherwise stated.

Expression Vectors.

The wt-$hPAR_2$ cDNA possessing a C-terminal HA11 epitope (YPYDVPDYA) used in this study has been described in detail previously [1]. Human PAR_2C361A was generated using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands), according to the manufacturer's instructions. The engineered mutation in PAR_2 was then confirmed by automated DNA sequencing (MWG, Germany).

Cell culture and transfection

The Pro5 Chinese Hamster Ovary (CHO) cell line, that permanently expressed either wild-type or mutant $hPAR_2$ receptors, were propagated in selective growth media (α-MEM containing 10% FCS, 100 units/ml penicillin, 100 ng/ml streptomycin, 250 ng/ml amphotericin B, and 800 µg/ml geneticin) 5% CO_2 at 37^{o}C. All cell lines were harvested using non-enzymatic cell dissociation solution.

Permanent receptor expressing cell lines cells were generated by transfection using the LipofectAMINE2000 (Invitrogen, Paisley, U.K.) method, according to the manufacturer's protocol. Transfected Pro5 cells were propagated in selective growth medium for two weeks to allow selection. Permanently expressing cell-lines were selected by single cell cloning and assessed for receptor expression by flow cytometry.

Determination of cell surface expression of $hPAR_2$ wild-type, and $hPAR_2$C361A receptors.

It has previously been found 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 Sam11 mouse anti-human PAR_2 antibody and anti-mouse FITC conjugate before analysing with flow cytometry to assess and match receptor expression.

Localisation of Receptor by Confocal Imaging

Confocal microscopy was used to determine the location of PAR_2 and PAR_2C361A within the cells. Sam11 shows cell surface expression and HA11 shows total cell expression. For Sam11 staining cells grown on coverslips were incubated with Sam11 anti-PAR_2 antibody (1µg/ml) at RT for 60 min before incubation with FITC conjugated anti-mouse antibody (1µg/ml). For HA11 staining, coverslips were pre-incubated in PBS (1% BSA, 3% Rabbit Serum) for 15 min prior to addition of anti-HA11 mouse monoclonal antibody (1µg/ml) and incubated at RT for 60 min. Coverslips were then incubated

with FITC conjugated anti-mouse antibody (1μg/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 photo being chosen.

[^{3}H]-palmitate labelling and PAR_2 immunoprecipitation.

The constitutive palmitoylation of PAR_2 was investigated by detecting [^{3}H]-palmitate labelling of the receptor. Wt-hPAR_2 and hPAR_2C361A expressing Pro5 cell lines were seeded in 100 mm dishes, pre-incubated overnight in serum-free medium, and labelled with 1 mCi [^{3}H] palmitic acid for 4 h at 37 °C. After labelling, PAR_2 protein from the cell lines was isolated by immunoprecipitation using the μMACS™ HA11 Epitope Tag Protein Isolation Kit (Miltenyi Biotech, Bergisch, Germany) as per manufacturers protocol.

Proteins were then resolved on SDS-PAGE in non-reducing conditions and then fixed for 30 min (isopropanol:water:acetic acid, 25:62:10). Gels were then treated with Amplify™ for 15 min at RT before drying under vacuum at 80^{o}C. The dried gel was then exposed to Hyperfilm™ x-ray film at -80^{o}C for 24 hours, before developing.

In order to confirm equal loading of radiolabelled PAR_2 protein, immunoblots were performed with half of the protein sample used in the palmitate experiments. Briefly immunoprecipitated samples were separated on a 10% SDS/PAGE gel before transfer to Hybond C PVDF membrane. The membrane was blocked with 5% non-fat milk powder in PBS/Tween 20 (0.1%) for 1 h before incubation overnight at 4 °C with the mouse monoclonal anti-HA.11 antibody (1 in 1000 dilution in PBS/Tween-20 (0.1%) containing 2% non-fat milk) then incubated with peroxidase conjugated goat anti-mouse IgG (1 in 1000) for 1 h. The epitope tagged hPAR_2 receptor was then visualized using enhanced chemiluminescence (ECL2), and captured on a UVP Epi Chem II Darkroom with 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 $CaCl_2$, 10 mM glucose, 20 mM Hepes and 0.25 mM sulphinpyrazone) pH 7.4. Fluorescence measurements were performed on a Photon Technology International Fluorospectrophotometer (West Sussex, UK), 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 calcium ionophore (A23187).

ERK1/2 phosphorylation and immunoblots.

Cells were grown in 6 well plates to a confluence of 40-50% in normal media. Media was then replaced with serum-free media and cells incubated overnight. Cells were then treated with agonist for a specified time (0, 5, 10, 30, 60 min) before halting treatment and harvesting cells in Laemlli's Sample Buffer (30% glycerol, 140mM Tris pH 6.8, 5% SDS, 7 nM Bromophenol blue, 5 mM EDTA, 1/20 2-mercaptoethanol). Samples were then incubated at 100^{o}C for 5 min prior to analysis by western blotting (as described in [^{3}H]-palmitate labelling and PAR_2 immunoprecipitation) using p44/42 MAP kinase Antibody (1 in 1000) and Phospho-p44/42 MAP Kinase Antibody (1 in 1000) (Cell Signaling Technology, NEB, Herts, UK).

Receptor Internalisation Assay

Semi-confluent cells (approx. 40% confluence) were harvested and treated with trypsin (100 nM) or the synthetic peptide SLIGKV-NH_2 (100 μM) which corresponds to the tethered ligand exposed by trypsin cleavage of PAR_2, for 0-30min at RT before being placed on ice. Cells were then washed and centrifuged. Pelleted cells were resuspended in cold-PBS and placed on ice for 10 min before incubation with Sam11 mouse anti-PAR_2 monoclonal antibody (3 μg/mL) for 1 h on ice followed by incubation with anti-mouse FITC-conjugated antibody (10 μg/mL) for a further 45 min. Cells were then analysed for PAR_2 cell-surface expression by flow cytometry (Becton Dickinson, Oxford, U.K.).

Statistical Analysis.
Graphs were produced using Prism Graphpad. For a two dataset comparison of group mean a two-tailed test was used, when this was to compare two different treatments on the same cell line, or when this was to compare two different cell lines subjected to the same treatment a paired t-test was adopted. In order to assess a change over a period of time using a specific treatment, or to assess a change over a concentration range for a specific cell line a repeated measures one-way ANOVA table was used. When a comparison of two different time courses or concentration ranges was required a two-way ANOVA table was adopted.

RESULTS

Expression and palmitoylated state of wt-PAR_2 and PAR_2C361A.

In order to study the potential role of palmitoylation in regulating PAR_2 function, Pro5 cells were stably transfected with HA11-tagged wt-hPAR_2 or hPAR_2C361A where the putative palmitoylation site (C361) had been replaced with an alanine. 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 localisation of receptor expression (Figure 3). When stained with Sam11 anti-PAR_2 antibody empty vector transfected cells (Figure 3A) showed no staining. Wt-hPAR_2 (Figure 3B) and hPAR_2C361A (Figure 3C) cells showed staining at the cell membrane. However, low levels of fluorescence were seen using this method and a high photomultiplier gain was required to observe any signal. Cells were therefore permeabilised and stained with anti-HA11 antibody. Wt-hPAR_2 (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 hPAR_2C361A (Figure 3F) was strikingly different to that of wt-hPAR_2 (Figure 3E). The ring staining for hPAR_2C361A 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 hPAR_2C361A appeared brighter than that of wt-PAR_2.

The palmitoylation state of the wt-hPAR_2 and hPAR_2C361A was then determined by metabolic labelling of the cells with [^{3}H] palmitic acid followed by immunoprecipitation of the receptors. Figure 4A shows that detectable levels of tritium were incorporated into wt-PAR_2. Two broad smears were observed, one between ~50-75kDa and a second from ~25-30kDa. In contrast, PAR_2C361A displayed an undetectable level of tritium incorporation. Western blot analysis of the same samples that were employed for the palmitoylation gel, demonstrated that approximately equal amounts of PAR_2 protein were present in the wt-PAR_2 and PAR_2C361A samples (Figure 4B).

Role of palmitoylation in regulating intracellular calcium signalling.

To assess the importance of palmitoylation in regulating hPAR_2 coupling to calcium, we compared the ability of wt-hPAR_2 and hPAR_2C361A to trigger increases in intracellular calcium in response to the hPAR_2 agonists trypsin and SLIGKV-NH_2. Trypsin and SLIGKV-NH_2 concentration effect curves for hPAR_2 and hPAR_2C361A cell lines with matched expression (Figure 2) were constructed (Figure 5). For wt-hPAR_2, both trypsin and SLIGKV-NH_2 stimulated robust and similar maximal responses (~60% of A23187) at 316 nM, and 316 μM respectively (Figure 5). The EC_{50} for trypsin and SLIGKV-NH_2 in activating wt-PAR_2 was 11.44 nM and 15.69 μM respectively. In contrast to wt-hPAR_2, trypsin and SLIGKV-NH_2 displayed significantly reduced efficacy towards hPAR_2C361A ($P=0.0003$ and $P=0.0058$ respectively). The maximal obtainable responses to both trypsin and SLIGKV-NH_2 were only ~50% and ~30% of that observed for wt-hPAR_2 respectively (Figure 5). The hPAR_2C361A EC_{50}s for trypsin and SLIGKV-NH_2 were 40.76 nM and 103.45 μM respectively, displaying a 4 and 6 fold increase compared to that obtained for wt-hPAR_2.

Inhibition of Trypsin Mediated Calcium Signal by Pertussis Toxin.

Pertussis toxin was used to ascertain what proportion of the total IP_3 induced Ca^{2+} flux is due to signalling through G_i in wt-hPAR_2 and the palmitoylation deficient mutant hPAR_2C361A. PTX dose

dependently inhibited wt-hPAR$_2$ and hPAR$_2$C361A mediated calcium signalling to trypsin. wt-hPAR$_2$ 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). Whereas, hPAR$_2$C361A produced a maximum calcium signal of ~30% of A23187 reducing down to <10% of A23187 at the maximum PTX concentration of 316 ng/ml. As such both curves reduce by ~20% of their A23187 signal (Figure 6).

Role of Palmitoylation in Regulating ERK1/2 Phosphorylation.

Having established that the palmitoylation deficient hPAR$_2$C361A mutant receptor coupled less efficiently to calcium, we asked whether receptor palmitoylation possibly regulated hPAR$_2$ signalling to ERK1/2 (p44/42). Significant differences in the pattern of the ERK1/2 stimulation were observed for hPAR$_2$C361A compared to wt-hPAR$_2$ ($p<0.001$) (Figure 7A). SLIGKV-NH$_2$ stimulated a significantly greater degree of ERK1/2 phosphorylation in hPAR$_2$C361A 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 hPAR$_2$C361A compared to wt-hPAR$_2$ ($P< 0.001$) (Figure 7B). Trypsin stimulated a significantly greater degree of ERK1/2 phosphorylation in hPAR$_2$C361A cells at the 5, 10 and 20 min time points, and a more sustained ERK1/2 activation levels remaining significantly elevated up to the 60 min time point tested. All phosphorylated ERK1/2 blots were stripped of antibodies and re-probed with anti-total ERK1/2 and the phosphorylated ERK1/2 were then normalised against total ERK1/2 and expressed as a fold change of no treatment (NT).

Inhibition of MAP Kinase Signalling by Pertussis Toxin

To ascertain what proportion of the ERK signalling is due to signalling through G_i and to determine whether the lack of palmitoylation altered this signalling, wt-hPAR$_2$, hPAR$_2$C361A and empty vector control cells were preincubated with PTX before treating with PAR$_2$ agonists (trypsin and SLIGKV).

Trypsin and SLIGKV-NH$_2$ evoked negligible changes in ERK1/2 signalling compared to NT in the empty vector cells (data not shown). Treatment with PTX caused no significant change in ERK1/2 agonist induced signalling. For wt-hPAR$_2$ (Figure 8A) SLIGKV-NH$_2$ 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 though no significant difference was noted. SLIGKV-NH$_2$ and trypsin evoked a 2 and 3 fold increase in ERK1/2 signalling over NT respectively in hPAR$_2$C361A expressing cells (Figure 8B). When pre-treated with PTX ERK1/2 signalling in hPAR$_2$C361A decreased a small amount following no treatment and trypsin treatment in comparison to samples not pre-treated with PTX. However, a large drop in ERK1/2 signalling can be seen in the PTX pre-incubated SLIGKV-NH$_2$ treated samples compared those not pre-incubated with PTX, though the changes are not statistically significant.

Agonist Triggered Internalisation of PAR$_2$.

Flow cytometry was employed to determine the agonist induced internalisation of PAR$_2$ using the monoclonal Sam11 antibody which recognises 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-PAR$_2$ by 10 min post agonist challenge. At the 60 min time point ~42% of wt-PAR$_2$ was still detectable at the cell surface. Curiously, SLIGKV-NH$_2$ (100 μM) only stimulated a modest loss of cell surface receptor at 30 and 60 min post agonist challenge. In contrast to wt-hPAR$_2$, trypsin stimulated a significantly greater loss of hPAR$_2$C361A from the cell surface at all 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-hPAR$_2$ cells, we also observed only a minor degree of receptor internalisation with hPAR$_2$C361A following treatment with SLIGKV-NH$_2$ with maximal internalisation observed at 60 min (16 ± 2%).

DISCUSSION

The data presented here provides the first direct biochemical and molecular evidence that $hPAR_2$ is palmitoylated and that cysteine 361 is likely to be the primary site of palmitoylation. In addition evidence is presented here which demonstrates that palmitoylation of $hPAR_2$ is a dynamic process that is influenced by agonist activation. Further, palmitoylation of $hPAR_2$ was shown to regulate constitutive receptor expression, agonist triggered internalisation and more importantly inversely regulate receptor signalling to two major signalling pathways, namely calcium and MAPK.

The $hPAR_2C361A$ cell line displayed greater receptor expression than wt-$hPAR_2$. Removal of palmitoylation often results in loss of cell surface expression as seen with the V_2 vasopressin receptor and CCR5 [15-17]. To our knowledge, this is the first study to find an increase in receptor expression resulting from the mutagenic removal of a palmitoylated cysteine. Possible reasons for increased receptor expression are covered later.

Confocal microscopy was next employed to compare the cellular distribution of wt-$hPAR_2$ and $hPAR_2C361A$. Confocal imaging shows that PAR_2 is located at the plasma membrane along with some internal localisation (which is greater in $hPAR_2C361A$), this is presumably, at least in part, PAR_2 contained in golgi stores, which is confirmatory of previous confocal imaging of PAR_2 [18-20].

Immunoprecipitation of wt-$hPAR_2$ and $hPAR_2C361A$ was carried out using the HA11 epitope tag, and western blotting carried out using anti-HA11 antibody as Sam11 does not work well with western blotting. Immunoprecipitation was required as half of the sample was used to detect palmitoylation, it was therefore essential to remove unwanted (non-HA11 tagged) proteins. The banding pattern seen is in keeping with the previous immunoblotting of $hPAR_2$ [1] and the C361A appears to have no perceptible effect on the receptor molecular weight compared to wild-type. Despite flow cytometry data showing higher $hPAR_2C361A$ expression than the wild type, three times more cells were required to achieve matching quantities of receptor. However, if $hPAR_2$ is palmitoylated at C361, 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 so 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 $hPAR_2$ 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 $hPAR_2C361A$ it seems likely this is the sole palmitoylation site for $hPAR_2$.

PAR_2 palmitoylation has a significant effect on receptor mediated calcium signalling. The decreased signal and agonist sensitivity observed for the $hPAR_2C361A$ 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 appears to allow agonist-independent phosphorylation of receptor PKA and GRK sites which would normally only phosphorylate upon activation [24]. In other GPCRs desensitisation/internalisation can be driven by phosphorylation dependent interations with β-arrestin [24-26]. Mutagenic replacement of the $hPAR_2$ palmitoylation site may alternatively cause conformational change in the receptor leaving the tethered ligand and AP incapable of correctly binding to the ligand binding site. Although this has not previously been seen in the mutagenic replacement of palmitoylation, removal and mutagenesis of areas of the C-terminal tail in some GPCR 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 are 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 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 PAR_2 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 PAR_2 results in an increased affinity for β-arrestin binding which in turn uncouples the receptor from it's G-protein by a steric mechanism [18-20]. Additionally, PAR_2 expressed alongside a

β-arrestin mutant incapable of receptor binding shows 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]. Recently PAR_2 activation has also been shown to promote β-arrestin actions-independent of G-proteins by direct inhibition of $G\alpha_{q/11}$, as well as receptor coupling to $G\alpha_{q/11}$ [35]. There is additional evidence to suggest that residues in the C-terminus of PAR_2 define 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 $hPAR_2C361A$ maybe enhanced due to an increase in β-arrestin association. This increased β-arrestin association may also go to explain the increased receptor expression seen in $hPAR_2C361A$ compared to wt-$hPAR_2$ as studies have shown a role for β-arrestins in trafficking of PAR_2 from the golgi [35, 38] Further studies to investigate the role of β-arrestins in $hPAR_2C361A$ signalling, expression and internalisation 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 $G\alpha_q$ interacts with caveolin, where as $G\alpha_i$ does not, suggesting they may therefore be targeted to different lipid regions [41]. The oxytocin receptor (OTR) is known to couple to both G_q and G_i but differentially activates one or the other depending on it's membrane localisation [42]. When located within lipid rafts OTR activation results in cell growth through a G_q mediated pathway, when outside lipid rafts it results in G_i mediated inhibition of cell growth. This effect on cell growth is shown to be as a result of a different temporal pattern of EGFR and ERK1/2 phosphorylation, and is shown to be more persistent when receptors are located outside of lipid raft microdomains [42]. As such palmitoylation in $hPAR_2$ may target the receptor to specific lipid raft microdomains, removal of which results in movement outside of these lipid rafts resulting in greater G_i association and prolonged ERK1/2 signalling.

Palmitoylation has been shown to affect a GPCR ability to couple to its respective G-protein α subunit. For some GPCRs palmitoylation has been shown to selectively effect coupling to specific Gα subunits [29, 32] removing coupling to one or more subunits whilst maintaining coupling with another. PTX, an inhibitor of signalling through G_i was used to further understand the effect of palmitoylation on PAR_2 G-protein coupling calcium signalling. Interestingly calcium signalling of wt-$hPAR_2$ and $hPAR_2C361A$ was reduced by a similar magnitude, however in the mutant receptor, this reduction almost completely ablated the trypsin evoked calcium response suggesting signalling in $hPAR_2C361A$ is due to coupling with G_i. Although previous GPCRs have shown selective uncoupling of Gα subunits [29, 32] these have preferentially lost G_i and maintained G_q coupling. This was suggested by Okomoto et al as being due to G_i requiring a higher level of palmitoylation. However, if palmitoylation of $hPAR_2$ targets the receptor to G_q containing lipid rafts, as with the OTR [42], and removal of palmitoylation results in the receptor locating outside of the raft then association with G_q would be disrupted. A recent study suggested that PAR_2, unlike PAR_1, does not signal through G_i [43], however in our cell system, it appears that in order to provoke calcium signalling $hPAR_2C361A$ couples to G_i. Like DeFea et al we showed that $hPAR_2$ activates ERK1/2 through a PTX insensitive pathway, so having little or no involvement by G_i [18]. In contrast, the non palmitoylated $hPAR_2C361A$ displayed reduced ERK phosphorylation post SLIGKV-NH_2 activation, suggesting a G_i mediated response. Numerous studies have shown ERK activation to be independent of G-protein signalling and PAR_2 ERK signalling has been shown to be mediated by β-arrestin association [35, 38]. It maybe the increased association with G_i results in a shift in the preferred pathway for ERK1/2 activation. The signalling pathway responsible for activation of ERK in PAR_2 has previously been shown to "switch" in the case of PAR_2δST363/366A, a phosphorylation deficient mutant, which activated ERK1/2 via a proline-rich tyrosine kinase-2 (PYK2) mediated pathway [18]. It was hypothesized that the interaction with the PYK2 pathway was a result of the prolonged Ca^{2+}-mobilisation observed in this mutant. As stated earlier Ca^{2+}-mobilisation following $hPAR_2C361A$ activation takes longer to reach a signalling plateau so it maybe that the G_i-dependant activation of ERK1/2 activation observed is PYK2-mediated. In addition to G_i and G_q, PAR_2 is also thought to signal through $G_{12/13}$ and Rho pathways [43]. Although we have not investigated these pathways here it would be interesting to determine the effect of PAR_2 palmitoylation status on these pathways.

The rate and extent of internalisation in response to trypsin is increased in the palmitoylated mutant compared to wt-$hPAR_2$. However, neither mutant nor wild-type receptor showed

internalisation following treatment with SLIGKV-NH_2. This is contrary to what has previously been reported for PAR_2 following SLIGKV-NH_2 treatment [18-20]. Internalisation rather than receptor degradation of PAR_2 was confirmed by permeabolising cells and using endocytotic and PKC inhibitors concanavalin A and hyperosmolar glucose as well as fixing the membrane (data 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 $hPAR_2$ internalises by clathrin-coated pits, and $hPAR_2$C361A internalises by the same mechanism albeit more rapidly. Although this study is the first to report a differential effect of receptor cell surface expression and post-agonist endocytosis with trypsin and SLIGKV-NH_2, the internalisation seen post-SLIGKV-NH_2 treatment in previous publications maybe due to over-expression of β-arrestin in the cell lines used. A recent study has shown over-expression of β-arrestin to result in its constitutive association with cellular components [35].

Despite increased expression of $hPAR_2$C361A compared to PAR_2 in the resting state, with the addition of agonists the endocytosis of the mutant receptor appears increased over the wild type. This enhanced post-activation endocytosis may be for a number of different reasons. If as previously discussed $hPAR_2$C361A 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 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 $hPAR_2$ functions similarly to the OTR, then following activation the receptor depalmitoylates and needs to translocate from the caveolae domain before being endocytosed [42]. The removal of $hPAR_2$ palmitoylation would mean the receptor was already located outside of the caveolae 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 G_q, phospholipase C (PLC) activity has previously been shown to inhibit endocytosis via clathrin-coated pits [49]. Since $hPAR_2$C361A has reduced/abolished coupling to G_q, PLC activity would be significantly reduced/abolished so 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 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 previously been shown to be the rate limiting step for β-arrestin association [50]. As stated earlier 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 prove this by further experimentation.

This is the first study investigating the palmitoylation status of $hPAR_2$ and its effect on receptor function. We have demonstrated that $hPAR_2$ is palmitoylated pre-dominantly, if not entirely on C361. We have further demonstrated the multifaceted importance of palmitoylation on receptor expression, agonist sensitivity, densensitisation and internalisation.

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REFERENCES

1 Compton, S. J., Sandhu, S., Wijesuriya, S. J. and Hollenberg, M. D. (2002) Glycosylation of human proteinase-activated receptor-2 (hPAR2): role in cell surface expression and signalling. Biochem J. **368**, 495-505

2 Qanbar, R. and Bouvier, M. (2003) Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. Pharmacol Ther. **97**, 1-33
3 Krupnick, J. G. and Benovic, J. L. (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu Rev Pharmacol Toxicol. **38**, 289-319
4 Ma, L. and Pei, G. (2007) Beta-arrestin signaling and regulation of transcription. J Cell Sci. **120**, 213-218
5 Compton, S. J., Renaux, B., Wijesuriya, S. J. and Hollenberg, M. D. (2001) Glycosylation and the activation of proteinase-activated receptor 2 (PAR(2)) by human mast cell tryptase. Br J Pharmacol. **134**, 705-718
6 Bijlmakers, M. J. and Marsh, M. (2003) The on-off story of protein palmitoylation. Trends Cell Biol. **13**, 32-42
7 Escriba, P. V., Wedegaertner, P. B., Goni, F. M. and Vogler, O. (2007) Lipid-protein interactions in GPCR-associated signaling. Biochim Biophys Acta. **1768**, 836-852
8 Kawate, N. and Menon, K. M. (1994) Palmitoylation of luteinizing hormone/human choriogonadotropin receptors in transfected cells. Abolition of palmitoylation by mutation of Cys-621 and Cys-622 residues in the cytoplasmic tail increases ligand-induced internalization of the receptor. J Biol Chem. **269**, 30651-30658
9 Eason, M. G., Jacinto, M. T., Theiss, C. T. and Liggett, S. B. (1994) The palmitoylated cysteine of the cytoplasmic tail of alpha 2A-adrenergic receptors confers subtype-specific agonist-promoted downregulation. Proc Natl Acad Sci U S A. **91**, 11178-11182
10 Ponimaskin, E. G., Schmidt, M. F., Heine, M., Bickmeyer, U. and Richter, D. W. (2001) 5-Hydroxytryptamine 4(a) receptor expressed in Sf9 cells is palmitoylated in an agonist-dependent manner. Biochem J. **353**, 627-634
11 Petaja-Repo, U. E., Hogue, M., Leskela, T. T., Markkanen, P. M., Tuusa, J. T. and Bouvier, M. (2006) Distinct subcellular localization for constitutive and agonist-modulated palmitoylation of the human delta opioid receptor. J Biol Chem. **281**, 15780-15789
12 Compton, S. J., Cairns, J. A., Palmer, K. J., Al-Ani, B., Hollenberg, M. D. and Walls, A. F. (2000) A polymorphic protease-activated receptor 2 (PAR2) displaying reduced sensitivity to trypsin and differential responses to PAR agonists. J Biol Chem. **275**, 39207-39212
13 Moffett, S., Mouillac, B., Bonin, H. and Bouvier, M. (1993) Altered phosphorylation and desensitization patterns of a human beta 2-adrenergic receptor lacking the palmitoylated Cys341. Embo J. **12**, 349-356
14 Molino, M., Barnathan, E. S., Numerof, R., Clark, J., Dreyer, M., Cumashi, A., Hoxie, J. A., Schechter, N., Woolkalis, M. and Brass, L. F. (1997) Interactions of mast cell tryptase with thrombin receptors and PAR-2. J Biol Chem. **272**, 4043-4049
15 Sadeghi, H. M., Innamorati, G., Dagarag, M. and Birnbaumer, M. (1997) Palmitoylation of the V2 vasopressin receptor. Mol Pharmacol. **52**, 21-29
16 Percherancier, Y., Planchenault, T., Valenzuela-Fernandez, A., Virelizier, J. L., Arenzana-Seisdedos, F. and Bachelerie, F. (2001) Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. J Biol Chem. **276**, 31936-31944
17 Blanpain, C., Wittamer, V., Vanderwinden, J. M., Boom, A., Renneboog, B., Lee, B., Le Poul, E., El Asmar, L., Govaerts, C., Vassart, G., Doms, R. W. and Parmentier, M. (2001) Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways. J Biol Chem. **276**, 23795-23804
18 DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D. and Bunnett, N. W. (2000) beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. J Cell Biol. **148**, 1267-1281

19 Dery, O., Thoma, M. S., Wong, H., Grady, E. F. and Bunnett, N. W. (1999) Trafficking of proteinase-activated receptor-2 and beta-arrestin-1 tagged with green fluorescent protein. beta-Arrestin-dependent endocytosis of a proteinase receptor. J Biol Chem. **274**, 18524-18535
20 Bohm, S. K., Khitin, L. M., Grady, E. F., Aponte, G., Payan, D. G. and Bunnett, N. W. (1996) Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. J Biol Chem. **271**, 22003-22016
21 Bockaert, J., Fagni, L., Dumuis, A. and Marin, P. (2004) GPCR interacting proteins (GIP). Pharmacol Ther. **103**, 203-221
22 Bockaert, J., Roussignol, G., Becamel, C., Gavarini, S., Joubert, L., Dumuis, A., Fagni, L. and Marin, P. (2004) GPCR-interacting proteins (GIPs): nature and functions. Biochemical Society transactions. **32**, 851-855
23 Hawtin, S. R., Tobin, A. B., Patel, S. and Wheatley, M. (2001) Palmitoylation of the vasopressin V1a receptor reveals different conformational requirements for signaling, agonist-induced receptor phosphorylation, and sequestration. J Biol Chem. **276**, 38139-38146
24 Moffett, S., Rousseau, G., Lagace, M. and Bouvier, M. (2001) The palmitoylation state of the beta(2)-adrenergic receptor regulates the synergistic action of cyclic AMP-dependent protein kinase and beta-adrenergic receptor kinase involved in its phosphorylation and desensitization. J Neurochem. **76**, 269-279
25 Munshi, U. M., Peegel, H. and Menon, K. M. (2001) Palmitoylation of the luteinizing hormone/human chorionic gonadotropin receptor regulates receptor interaction with the arrestin-mediated internalization pathway. Eur J Biochem. **268**, 1631-1639
26 Moffett, S., Adam, L., Bonin, H., Loisel, T. P., Bouvier, M. and Mouillac, B. (1996) Palmitoylated cysteine 341 modulates phosphorylation of the beta2-adrenergic receptor by the cAMP-dependent protein kinase. J Biol Chem. **271**, 21490-21497
27 Piersen, C. E., True, C. D. and Wells, J. N. (1994) A carboxyl-terminally truncated mutant and nonglycosylated A2a adenosine receptors retain ligand binding. Mol Pharmacol. **45**, 861-870
28 Klinger, M., Kuhn, M., Just, H., Stefan, E., Palmer, T., Freissmuth, M. and Nanoff, C. (2002) Removal of the carboxy terminus of the A2A-adenosine receptor blunts constitutive activity: differential effect on cAMP accumulation and MAP kinase stimulation. Naunyn-Schmiedeberg's archives of pharmacology. **366**, 287-298
29 Okamoto, Y., Ninomiya, H., Tanioka, M., Sakamoto, A., Miwa, S. and Masaki, T. (1997) Palmitoylation of human endothelinB. Its critical role in G protein coupling and a differential requirement for the cytoplasmic tail by G protein subtypes. J Biol Chem. **272**, 21589-21596
30 Hayashi, M. K. and Haga, T. (1997) Palmitoylation of muscarinic acetylcholine receptor m2 subtypes: reduction in their ability to activate G proteins by mutation of a putative palmitoylation site, cysteine 457, in the carboxyl-terminal tail. Arch Biochem Biophys. **340**, 376-382
31 Palmer, T. M. and Stiles, G. L. (2000) Identification of threonine residues controlling the agonist-dependent phosphorylation and desensitization of the rat A(3) adenosine receptor. Mol Pharmacol. **57**, 539-545
32 Doi, T., Sugimoto, H., Arimoto, I., Hiroaki, Y. and Fujiyoshi, Y. (1999) Interactions of endothelin receptor subtypes A and B with Gi, Go, and Gq in reconstituted phospholipid vesicles. Biochemistry. **38**, 3090-3099
33 Shenoy, S. K., Drake, M. T., Nelson, C. D., Houtz, D. A., Xiao, K., Madabushi, S., Reiter, E., Premont, R. T., Lichtarge, O. and Lefkowitz, R. J. (2006) beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. J Biol Chem. **281**, 1261-1273

34 Wei, H., Ahn, S., Shenoy, S. K., Karnik, S. S., Hunyady, L., Luttrell, L. M. and Lefkowitz, R. J. (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. Proc Natl Acad Sci U S A. **100**, 10782-10787
35 Zoudilova, M., Kumar, P., Ge, L., Wang, P., Bokoch, G. M. and Defea, K. A. (2007) beta -arrestin-dependent regulation of the cofilin pathway downstream of protease-activated receptor-2. J Biol Chem
36 Seatter, M. J., Drummond, R., Kanke, T., Macfarlane, S. R., Hollenberg, M. D. and Plevin, R. (2004) The role of the C-terminal tail in protease-activated receptor-2-mediated Ca2+ signalling, proline-rich tyrosine kinase-2 activation, and mitogen-activated protein kinase activity. Cell Signal. **16**, 21-29
37 Stalheim, L., Ding, Y., Gullapalli, A., Paing, M. M., Wolfe, B. L., Morris, D. R. and Trejo, J. (2005) Multiple independent functions of arrestins in the regulation of protease-activated receptor-2 signaling and trafficking. Mol Pharmacol. **67**, 78-87
38 Kumar, P., Lau, C., Wang, P., Mathur, M. and Defea, K. A. (2007) Differential effects of {beta}-arrestins on internalization, desensitization and ERK1/2 activation downstream of Protease Activated Receptor-2. Am J Physiol Cell Physiol
39 Melkonian, K. A., Ostermeyer, A. G., Chen, J. Z., Roth, M. G. and Brown, D. A. (1999) Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated. J Biol Chem. **274**, 3910-3917
40 Okamoto, T., Schlegel, A., Scherer, P. E. and Lisanti, M. P. (1998) Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. J Biol Chem. **273**, 5419-5422
41 Oh, P. and Schnitzer, J. E. (2001) Segregation of heterotrimeric G proteins in cell surface microdomains. G(q) binds caveolin to concentrate in caveolae, whereas G(i) and G(s) target lipid rafts by default. Mol Biol Cell. **12**, 685-698
42 Rimoldi, V., Reversi, A., Taverna, E., Rosa, P., Francolini, M., Cassoni, P., Parenti, M. and Chini, B. (2003) Oxytocin receptor elicits different EGFR/MAPK activation patterns depending on its localization in caveolin-1 enriched domains. Oncogene. **22**, 6054-6060
43 McCoy, K. L., Traynelis, S. F. and Hepler, J. R. (2010) PAR1 and PAR2 couple to overlapping and distinct sets of G proteins and linked signaling pathways to differentially regulate cell physiology. Mol Pharmacol. **77**, 1005-1015
44 Hansen, S. H., Sandvig, K. and van Deurs, B. (1993) Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. J Cell Biol. **121**, 61-72
45 Heuser, J. E. and Anderson, R. G. (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. J Cell Biol. **108**, 389-400
46 Lafleur, M. A., Mercuri, F. A., Ruangpanit, N., Seiki, M., Sato, H. and Thompson, E. W. (2006) Type I collagen abrogates the clathrin-mediated internalization of membrane type 1 matrix metalloproteinase (MT1-MMP) via the MT1-MMP hemopexin domain. J Biol Chem. **281**, 6826-6840
47 Jiang, A., Lehti, K., Wang, X., Weiss, S. J., Keski-Oja, J. and Pei, D. (2001) Regulation of membrane-type matrix metalloproteinase 1 activity by dynamin-mediated endocytosis. Proc Natl Acad Sci U S A. **98**, 13693-13698
48 Moore, C. A., Milano, S. K. and Benovic, J. L. (2007) Regulation of receptor trafficking by GRKs and arrestins. Annual review of physiology. **69**, 451-482
49 Carvou, N., Norden, A. G., Unwin, R. J. and Cockcroft, S. (2007) Signalling through phospholipase C interferes with clathrin-mediated endocytosis. Cell Signal. **19**, 42-51

50 Krasel, C., Vilardaga, J. P., Bunemann, M. and Lohse, M. J. (2004) Kinetics of G-protein-coupled receptor signalling and desensitization. Biochemical Society transactions. **32**, 1029-1031

FIGURE LEGENDS

Figure 1. Model in order to illustrate placement of haemagglutinin epitope tag (HA.11) and the four cysteine residues of interest. C361 is shown as a putative palmitoylation site and C148 and C226 are shown as putative sites for a disulphide bridge.

Figure 2. Assessment of receptor cell surface expression at varying cell confluences. Pro5 cells stably expressing wt-hPAR_2 and hPAR_2 C361A, were grown to confluence levels of 20 – 70% before harvesting and labelling with Sam11 anti-PAR_2 antibody and anti-mouse FITC conjugate and analysing using flow cytometry. Results are expressed as the mean ± SEM of 4 separate experiments.

Figure 3. Confocal images of wt-hPAR_2 and hPAR_2C361A localisation within the cell. Cells were incubated with Sam11 anti-PAR_2 primary antibody and anti-mouse FITC conjugated secondary antibody. Coverslips were then mounted and analysed by confocal microscopy. (A) - pcDNA3.1 empty vector. (B) - wt-hPAR_2. (C) - hPAR_2C361A. Fixed and permeabilised cells were incubated with anti-HA11 primary antibody and anti-mouse FITC conjugated secondary antibody before analysing by confocal microscopy. (D) - pcDNA3.1 empty vector. (E) - Wt-hPAR_2. (F) - hPAR_2C361A. Presence of PAR_2 at the plasma membrane is indicated with arrowheads ⮝. PAR_2 contained internally is indicated with arrows ↑. Images shown are representative of three experiments

Figure 4. [^{3}H]- palmitate incorporation into wt-hPAR_2 and hPAR_2C361A. HA11-epitope immunoprecipitated protein preparations from empty vector, wt-hPAR_2 and hPAR_2C361A cells each labelled with 1 mCi [^{3}H]-palmitate for 4 hours run on a 10% SDS-PAGE. Gel dried under vacuum and exposed to Hyperfilm™ x-ray film at -80^{o}C for 6 weeks (A). A portion of the same samples were analysed on western blot using anti-HA11 antibody to assess receptor loading (B) and a 20 min exposure time. Blots shown are representative of three experiments.

Figure 5. Agonist concentration effect curves for wt-hPAR_2 and hPAR_2 C361A. Fluo3 loaded Pro5 cells stably expressing for wt-hPAR_2 and hPAR_2C361A were stimulated with increasing concentrations of trypsin or SLIGKV-NH_2 and increases in intracellular calcium concentrations were measured. Results were expressed as a percentage of the maximum attainable response (response to A23187, calcium ionophore). Results are expressed as the mean ± SEM of 4 separate experiments. Each preformed in duplicate.

Figure 6. Graph showing inhibition of trypsin mediated Ca^{2+} release in wt-hPAR_2 and hPAR_2C361A. Changes in intracellular calcium levels were measured using a fluorospectrometer in fluo3 loaded wt- hPAR_2 and hPAR_2C361A Pro5 cells incubated with different concentrations of PTX (for 18 hours) prior to addition of 100 nM trypsin. Values shown as a percentage of the maximum attainable response (response to A23187).

Figure 7. Agonist stimulated MAPK phosphorylation curves and representative phosphorylated and total p44/42 western blots. Graphs showing densitometric values for bands produced by western blot for phosphorylated p44/42 post-SLIGKV-NH_2 treatment [A] and post trypsin treatment [B]. Normalised values are expressed as a fold change over no treatment (NT). Results are expressed as the mean ± SEM of 3 separate experiments, while the bands above are representative of the blots.

Figure 8. Western blots and bar charts showing inhibition of MAPK with PTX. wt- hPAR_2 (A), and hPAR_2C361A (B) cells were incubated with and without 100 ng/ml PTX (for 18 hours) prior to addition of 20 nM trypsin (Tryp) or 100 μM SLIGKV-NH_2 (KV) or no treatment (NT) for 10 mins.

Cells were lysed using laemlli buffer before separating by 10% SDS-PAGE and immunoblotting. Normalised values are expressed as a fold change over no treatment (NT). A representative total p44/42 and anti-phospho p44/42 blot is shown above the respective histogram. Results expressed as the mean ± SEM of 3 separate experiments.

Figure 9. Time course of receptor cell surface expression post-agonist addition. Pro5 cell stably expressing wt-hPAR_2 and hPAR_2 C361A were incubated with either 100 nM trypsin or 100 µM SLIGKV-NH_2 for increasing periods of time before washing and labelling with Sam11 anti-PAR_2 antibody and anti-mouse FITC conjugate and analysed using flow cytometry. Results are expressed as the mean ± SEM of 3 separate experiments

Figure 1

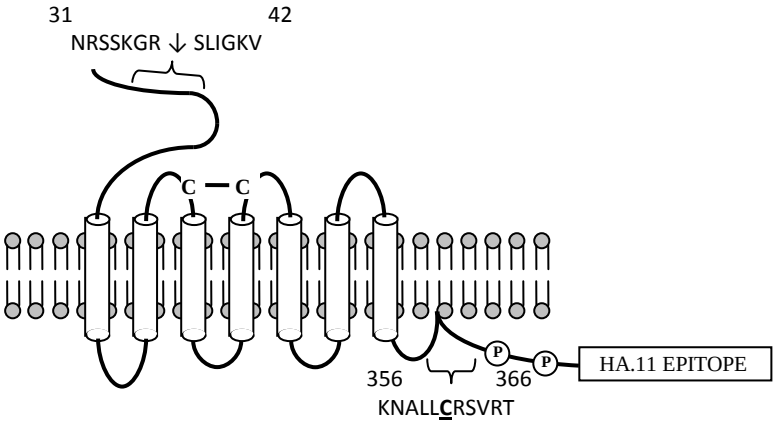


Figure 2

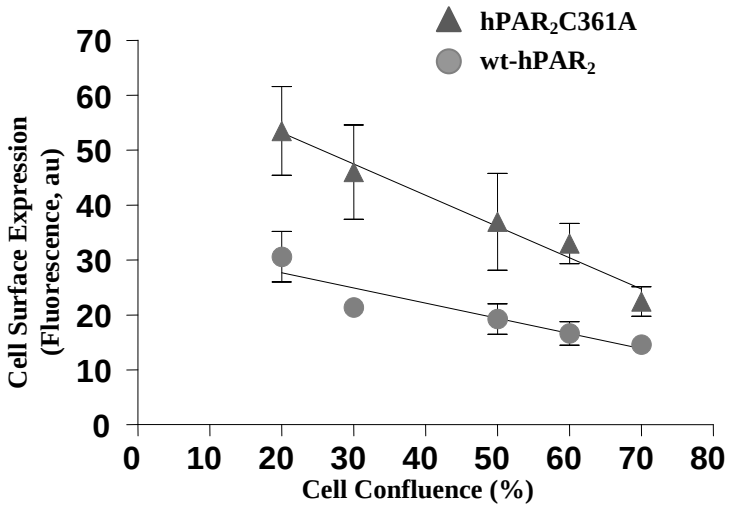

hPAR2C361A
wt-hPAR2
Cell Surface Expression
(Fluorescence, au)
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
Cell Confluence (%)

Figure 3

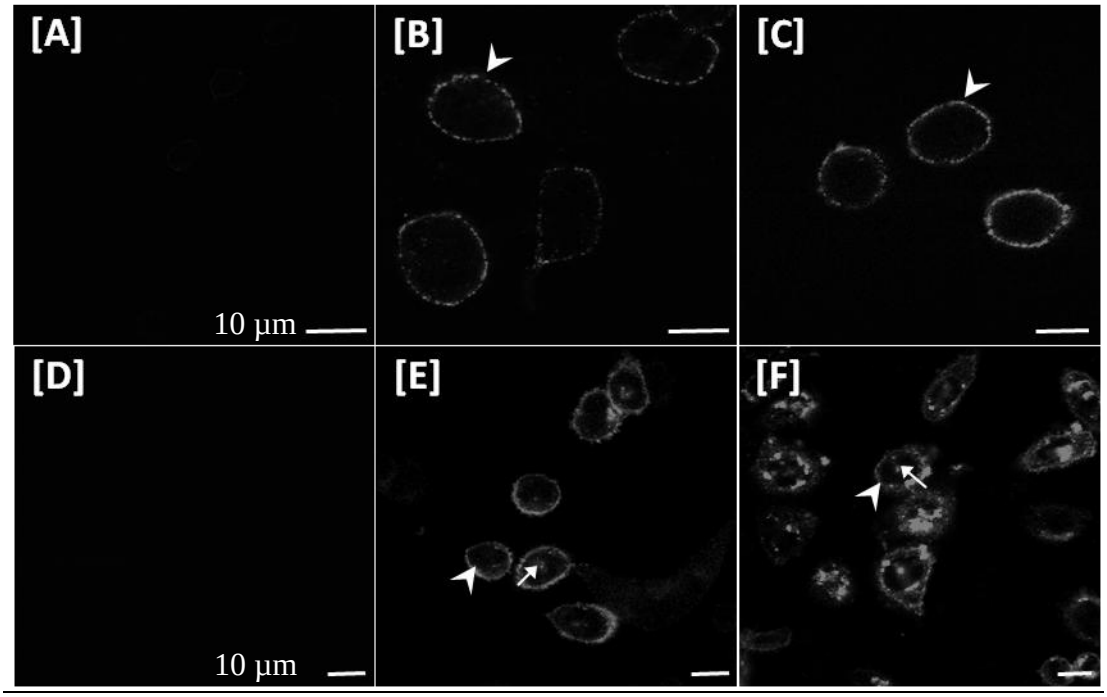


Figure 4

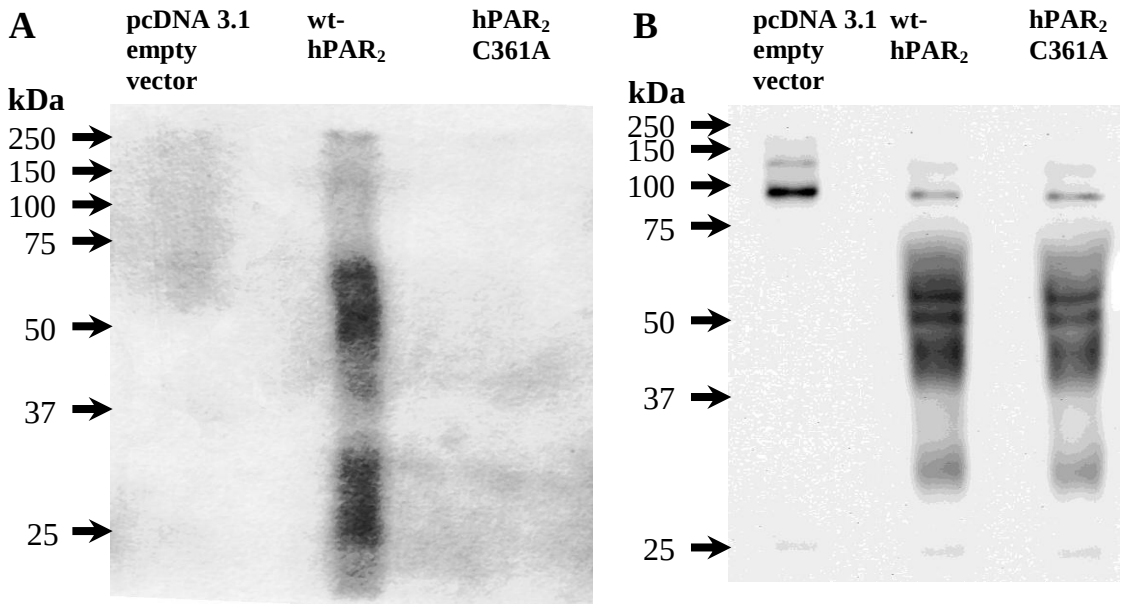

A
pcDNA 3.1 empty vector
wt-hPAR2
hPAR2 C361A
kDa
250
150
100
75
50
37
25
B
pcDNA 3.1 empty vector
wt-hPAR2
hPAR2 C361A
kDa
250
150
100
75
50
37
25

Figure 5

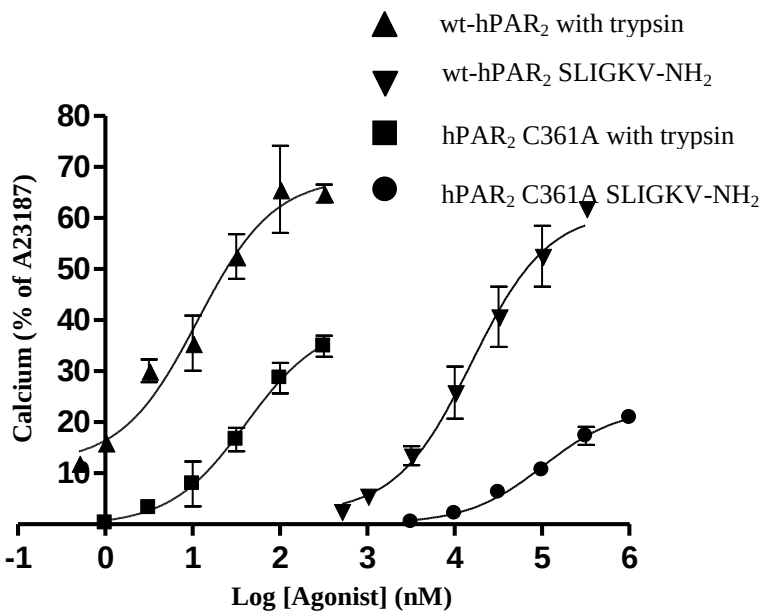

wt-hPAR2 with trypsin
wt-hPAR2 SLIGKV-NH2
hPAR2 C361A with trypsin
hPAR2 C361A SLIGKV-NH2
Calcium (% of A23187)
80
70
60
50
40
30
20
10
-1
0
1
2
3
4
5
6
Log [Agonist] (nM)

Figure 6

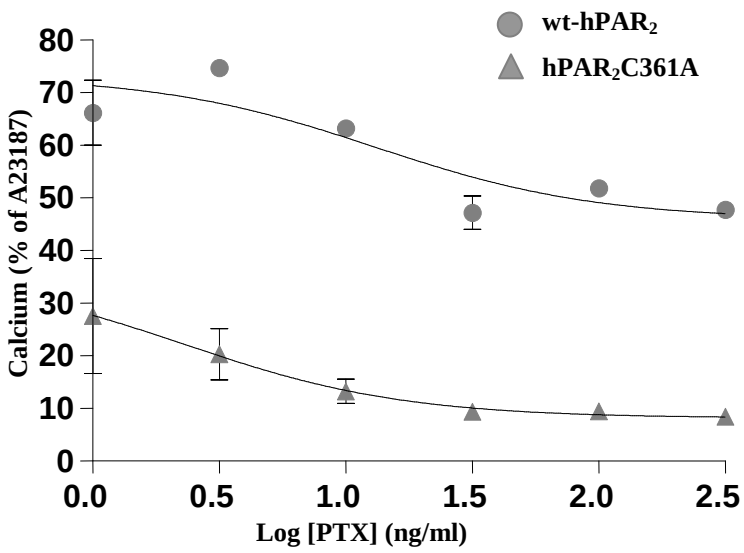

wt-hPAR2
hPAR2C361A
Calcium (% of A23187)
80
70
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
Log [PTX] (ng/ml)

Figure 7

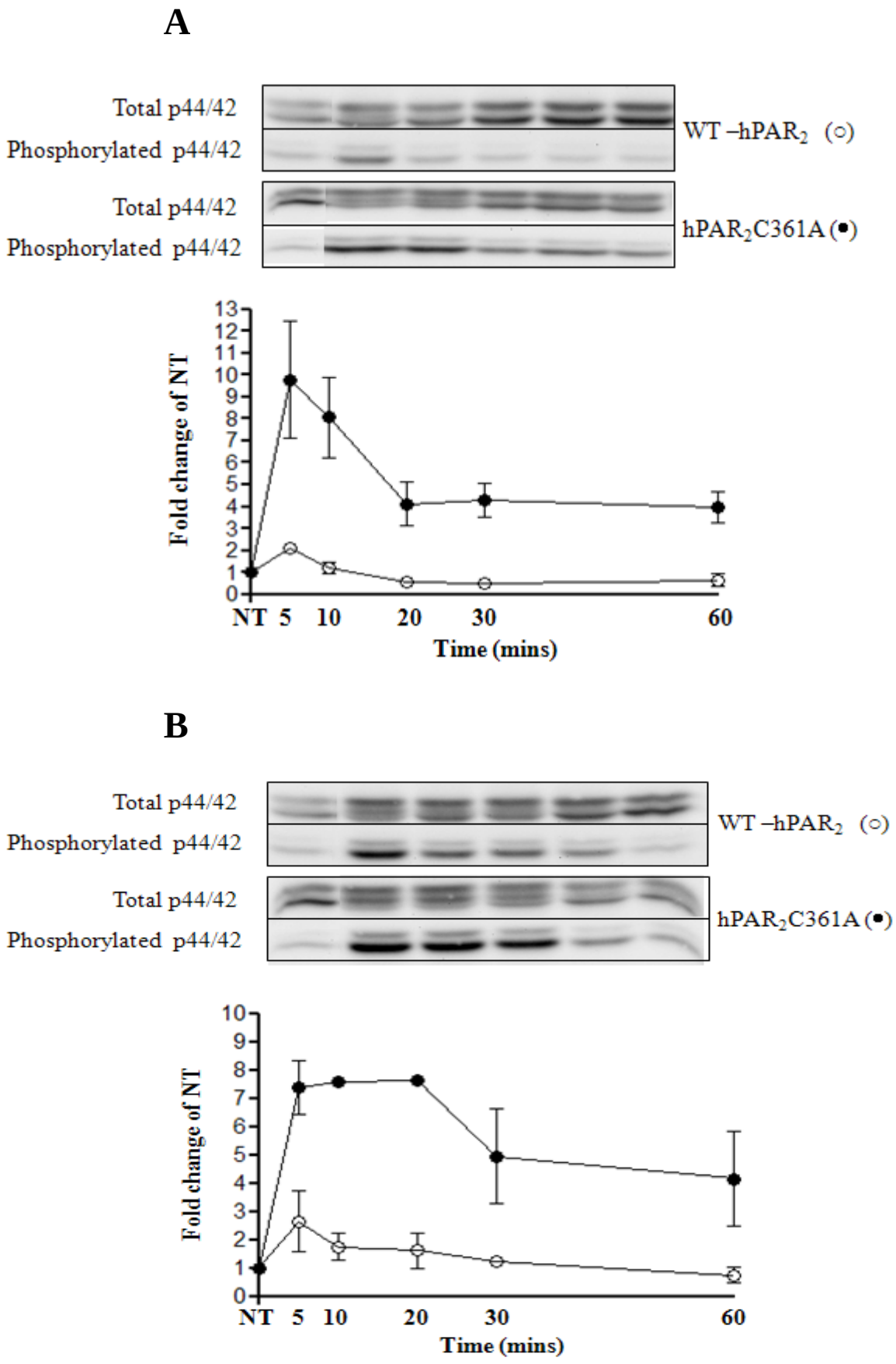

A
Total p44/42
Phosphorylated p44/42
WT –hPAR2 (○)
Total p44/42
Phosphorylated p44/42
hPAR2C361A (●)
Fold change of NT
0
1
2
3
4
5
6
7
8
9
10
11
12
13
NT 5 10 20 30 60
Time (mins)
B
Total p44/42
Phosphorylated p44/42
WT –hPAR2 (○)
Total p44/42
Phosphorylated p44/42
hPAR2C361A (●)
Fold change of NT
0
1
2
3
4
5
6
7
8
9
10
NT 5 10 20 30 60
Time (mins)

Figure 8

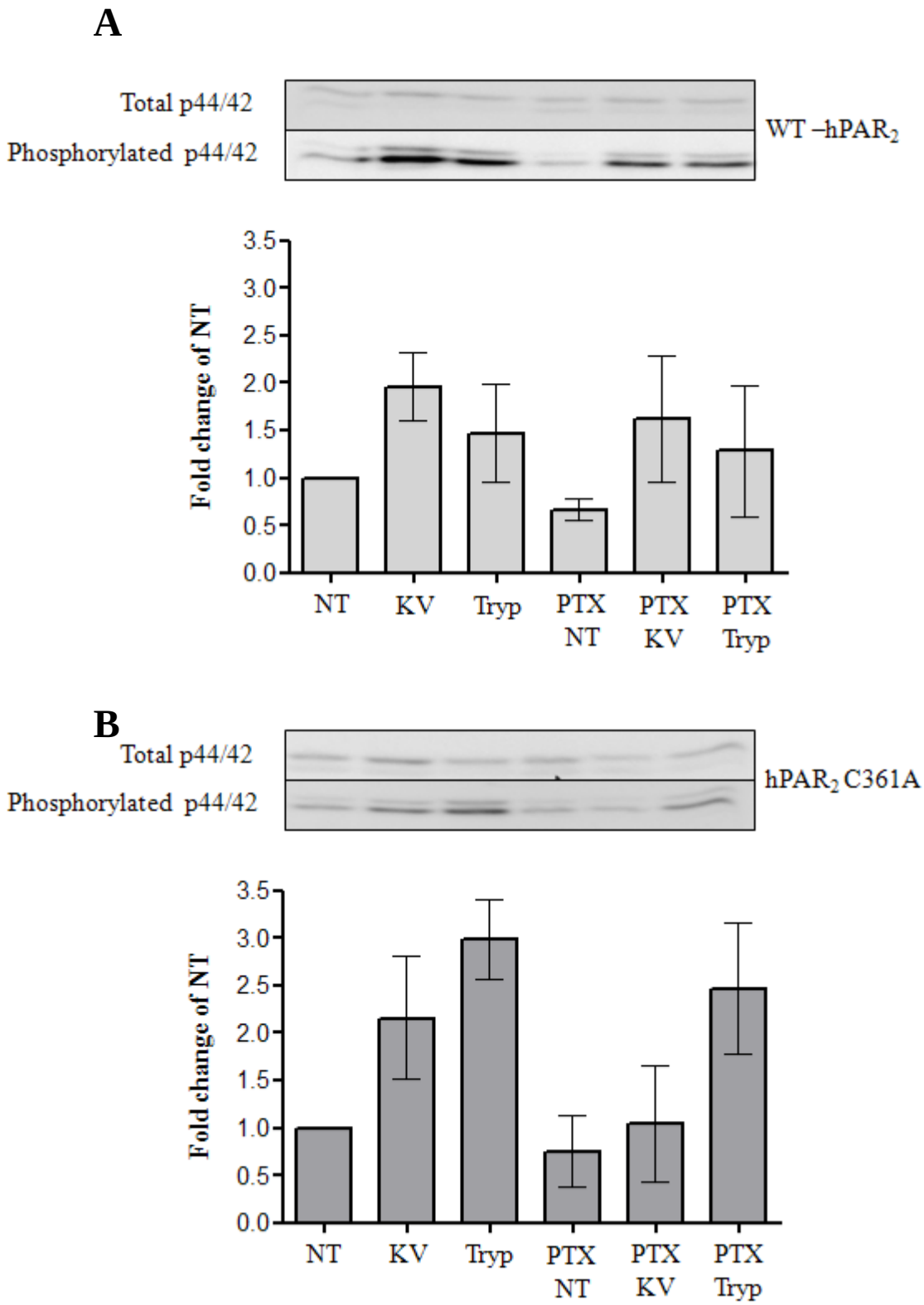
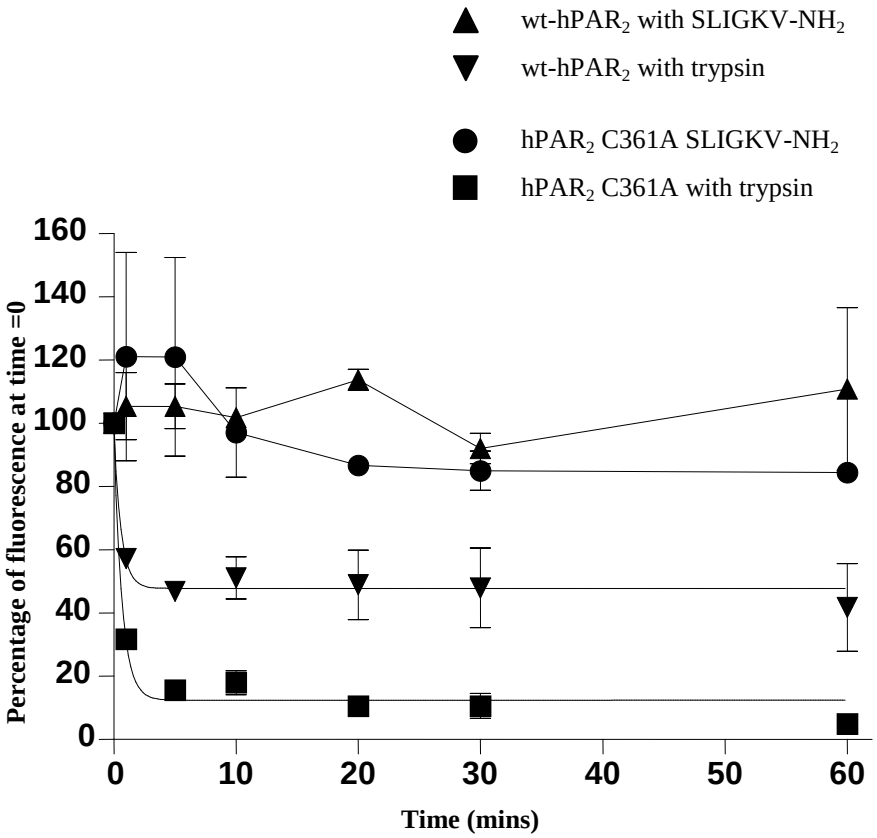

A
Total p44/42
Phosphorylated p44/42
WT –hPAR2
Fold change of NT
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NT
KV
Tryp
PTX NT
PTX KV
PTX Tryp
B
Total p44/42
Phosphorylated p44/42
hPAR2 C361A
Fold change of NT
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NT
KV
Tryp
PTX NT
PTX KV
PTX Tryp

Figure 9


wt-hPAR2 with SLIGKV-NH2
wt-hPAR2 with trypsin
hPAR2 C361A SLIGKV-NH2
hPAR2 C361A with trypsin
Percentage of fluorescence at time =0
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (mins)