Internalisation of membrane progesterone receptor-α after treatment with progesterone: Potential involvement of a clathrin-dependent pathway

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Abstract. Internalisation and recycling of seven transmembrane domain receptors is a critical regulatory event for their signalling. The mechanism(s) by which membrane progesterone receptor-α (mPRα) number is regulated on the cell surface is unclear. In this study, we investigated the cellular distribution of mPRα and mechanisms of mPRα trafficking using a cell line derived from a primary culture of human myometrial cells (M11) as an experimental model. RT-PCR and immunofluorescent analysis demonstrated expression of mPRα in M11 cells with mPRα primarily distributed on the cell surface under basal conditions. For the first time, plasma membrane localisation of mPRα was confirmed using immunogold transmission electron microscopy. Stimulation of M11 cells with progesterone (P4, 100 nM) resulted in internalisation of mPRα from the plasma membrane to the cytoplasm (10 min) and subsequent limited translocation back to the cell surface (20 min). We investigated potential endocytotic pathways involved in trafficking of mPRα after its internalisation. Partial co-localisation of clathrin with mPRα was obvious after 10 min of P4 treatment. Of note, chlorpromazine (inhibitor of clathrin-mediated pathway) inhibited the endocytosis of mPRα, whereas treatment with nystatin (inhibitor of caveolae-mediated pathway) did not affect internalisation. Collectively, these data suggest that mPRα is expressed on the cell surface of M11 cells and undergoes endocytosis after P4 stimulation primarily via a clathrin-mediated pathway.

Introduction

The large group of seven transmembrane domain receptors (7TMRs) include the well-known superfamily of G protein coupled receptors (GPCRs) as well as the phylogenetically distinct family of membrane progesterone receptors (mPRs) (1,2). Trafficking of 7TMRs between the various cell compartments is crucial for their ligand-binding and signalling functions. Internalisation of 7TMRs by endocytosis controls important receptor functions in the cell, fine tuning the binding characteristics of the receptor and regulating the activation of different signal transduction pathways. Some cell-surface receptors can also activate signal transduction pathways from intracellular compartments, suggesting that signalling can be regulated by compartmentalization (3).

Internalisation of many GPCRs involves rapid clathrin-mediated endocytosis (3-5). The agonist-occupied receptor is recruited into clathrin-coated pits (CCP), which then form vesicles for entry into the endocytic pathway. At this stage, vesicles and their cargo can be directed for either degradation or recycling back to the cell surface (6,7). For example, it has been shown that different internalisation routes seem to predetermine whether transforming growth factor β (TGF-β) receptors will trigger a signalling response or be degraded (3). The precise mechanisms influencing the fate of the CCP-receptor complex are not fully elucidated, but emerging data suggest that they are receptor specific and may vary between cell types (8). Another route by which GPCRs can be internalised involves caveolae and is independent of clathrin-coated pits (4,7). To date, nothing is known about the internalisation of another family of 7TMRs, the mPRs.

The mPRs were initially discovered in a teleost fish. More than 20 closely related genes have been cloned from other vertebrate species, including 3 mPR subtypes in humans, named α, β and γ, which show high levels of expression in human reproductive, brain and kidney tissues, respectively (1,2). Recently, ligand binding of another two members of the mPR family, named mPRδ and mPRε, has been characterised (9). Structural analyses of the translated cDNAs suggest that they encode membrane proteins with seven transmembrane domains. Thomas *et al* have shown that mPRα is a membrane-bound progestin receptor that activates G proteins, despite having a different ancestral origin to GPCRs (10). Karteris and co-workers have also shown that mPRα is expressed

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on myometrial cells and couples to inhibitory G proteins, resulting in an increased phosphorylation of myosin light chain (11). However, regulation of mPRα at the cell surface remains unclear, since there is a debate regarding the cellular distribution of these receptors. Therefore, in the present study we investigated the localisation of mPRα in M11 cells and potential endocytotic pathways involved in mPRα trafficking.

Materials and methods

Tissue culture. M11 cells were obtained from John A. Copland (Mayo Clinic College of Medicine, Jacksonville, FL, USA). These cells were derived from dispersed human myometrial cells by repeated passage without the use of any immortalising or transforming agents. M11 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose (with D-Valine) supplemented with 10% fetal bovine serum (FBS), 50 U penicillin and 50 μg streptomycin (Invitrogen, UK) and cultured at 37˚C in 5% CO_2.

RNA isolation, cDNA synthesis and RT-PCR. Total RNA was extracted using an RNA extraction kit (Sigma-Aldrich, UK) and treated with DNase I according to the manufacturer's instructions. RNA concentration was determined by spectrophotometric analysis (NanoDrop; Thermo Scientific, UK) and agarose gel electrophoresis. RNA (100 ng) was reverse-transcribed into cDNA using 5 IU/μl RNase H reverse transcriptase (Invitrogen). PCR amplification was carried out using Taq polymerase (Invitrogen) and oligonucleotide primers (Invitrogen) for human mPRα (Acc. AF313620). The primers for mPRα were forward 5'-GCTGTTCACTCACATCCC-3' and reverse 5'-TGGTGCAACCCCCAGA-3', resulting in a 289-bp PCR product. The primers for clathrin (138 bp) were forward 5'-TTAGCCGGTGCTGAAGAACT-3' and reverse 5'-CTGGAACCGACGGATAGTGT-3'. The primers for β-actin (216 bp) were forward 5'-AAGAGAGGCATCCTCACCCT-3' and reverse 5'-TACATGGCTGGGGTGTTGAA-3'. Thirty-three cycles for mPRα and 28 cycles for clathrin and β-actin were performed, consisting of an initial denaturing step at 94˚C for 30 sec followed by extension at 60˚C for 1 min and elongation at 72˚C for 1 min.

Treatment of M11 cells with progesterone (P4). Prior to P4 treatments, M11 cells were cultured in phenol red-free DMEM containing 2.5% charcoal-stripped FBS, penicillin and streptomycin, and incubated at 37˚C in 5% CO_2 for 24 h. Cells were exposed to 100 nM P4 for 0 (NS, unstimulated), 10 and 20 min.

Indirect immunofluorescence analyses using mPRα antibody. M11 cells were fixed in 4% paraformaldehyde for 10 min prior to washes in PBS and permeabilization with 0.2% Tween-20 for 20 min. After subsequent washes in PBS, samples were blocked for 1 h at room temperature using 10% bovine serum in PBS. Fixed M11 cells were then incubated with a 1:100 dilution of mPRα (hmPRα, 836-4) antibody in 1.5% bovine serum PBS overnight at 4˚C. The following day, preparations were washed with PBS prior to incubation with a 1:100 dilution of anti-rabbit IgG-fluorescein isothiocyanate (FITC)-conjugated antibody (Santa Cruz Biotechnology, USA) for 1 h. Sample preparations were washed with PBS and mounted in Vectashield® Mounting Medium (Vector labs) containing the DNA-specific dye 4,6-diamido-2-phenylindole (DAPI) to counterstain nuclei. Preparations were incubated with only secondary antibody as a negative control. Images were captured using a Plan Apo Neofluor x63 NA 1.25 oil objective (Zeiss; Carl Zeiss International) on a Zeiss Axiovert 200M microscope, and viewed using AxioVision software (Zeiss; Carl Zeiss International). Images were collected at set exposure times of 3000 msec, with the exception of the nuclei counterstained with DAPI, which were collected under an automatic exposure time.

Dual-indirect immunofluorescence using mPRα and clathrin antibodies. The general indirect immunofluorescence protocol described previously was adhered to, with several modifications. Samples were blocked for 1 h at room temperature in 10% bovine serum and 10% donkey serum in PBS. Preparations were incubated at 4˚C overnight in a 1:100 dilution of mPRα (hmPRα, 836-4) antibody and a 1:200 dilution of anti-clathrin heavy chain antibody (Abcam, UK) respectively diluted with 1.5% bovine serum and 1.5% donkey serum in PBS. The secondary antibody incubation consisted of a 1:100 dilution of anti-rabbit IgG-FITC antibody and a 1:100 dilution of anti-goat IgG rhodamine antibody (both from Santa Cruz Biotechnology, USA) respectively composed with 3% bovine serum and 3% donkey serum in PBS for 1 h.

Treatment of M11 cells with endocytosis inhibitors. The dual-indirect immunofluorescence protocol described previously using antibodies directed against mPRα and clathrin was used on M11 cells treated with the following inhibitors for various endocytotic pathways: chlorpromazine, dansylcadverine, nystatin and sucrose. M11 cells were cultured in phenol red-free DMEM containing 2.5% charcoal-stripped serum, penicillin and streptomycin, and incubated at 37˚C in 5% CO_2 24 h prior to inhibitor and P4 treatment. A negative control was prepared in each instance. M11 cells were incubated for 15 min at 37˚C with 50 μM chlorpromazine hydrochloride (Sigma-Aldrich) using a protocol described previously by Wang *et al* (12), then assessed under basal conditions (NS) and exposed to 100 nM P4 for 10 min. M11 cells were treated with 25 μg/ml nystatin (Sigma-Aldrich) at 37˚C for 30 min as previously described (13,14). Cells were subsequently exposed to 100 nM P4 for 10 min, but also treated under basal conditions (NS). M11 cells were incubated with 0.45 M sucrose (Sigma-Aldrich) at 37˚C for 15 min, described by Heuser and Anderson at basal conditions and prior to P4 exposure of 10 min (15).

Protein extraction from cultured M11 cells. M11 cells were cultured in 6-well dishes until reaching 80% confluency and maintained overnight in phenol red-free DMEM containing 2.5% charcoal-stripped FBS, penicillin and streptomycin, before the addition of agonists/inhibitors. Cells were lysed using 200 μl 2X Laemmli Buffer (Sigma-Aldrich) and denatured for 5 min at 100˚C.

Immunogold electron microscopy. M11 cells were grown to confluency on 35-mm polystyrene dishes at 37˚C in the presence of 5% CO_2 in high glucose DMEM with 10% heat-

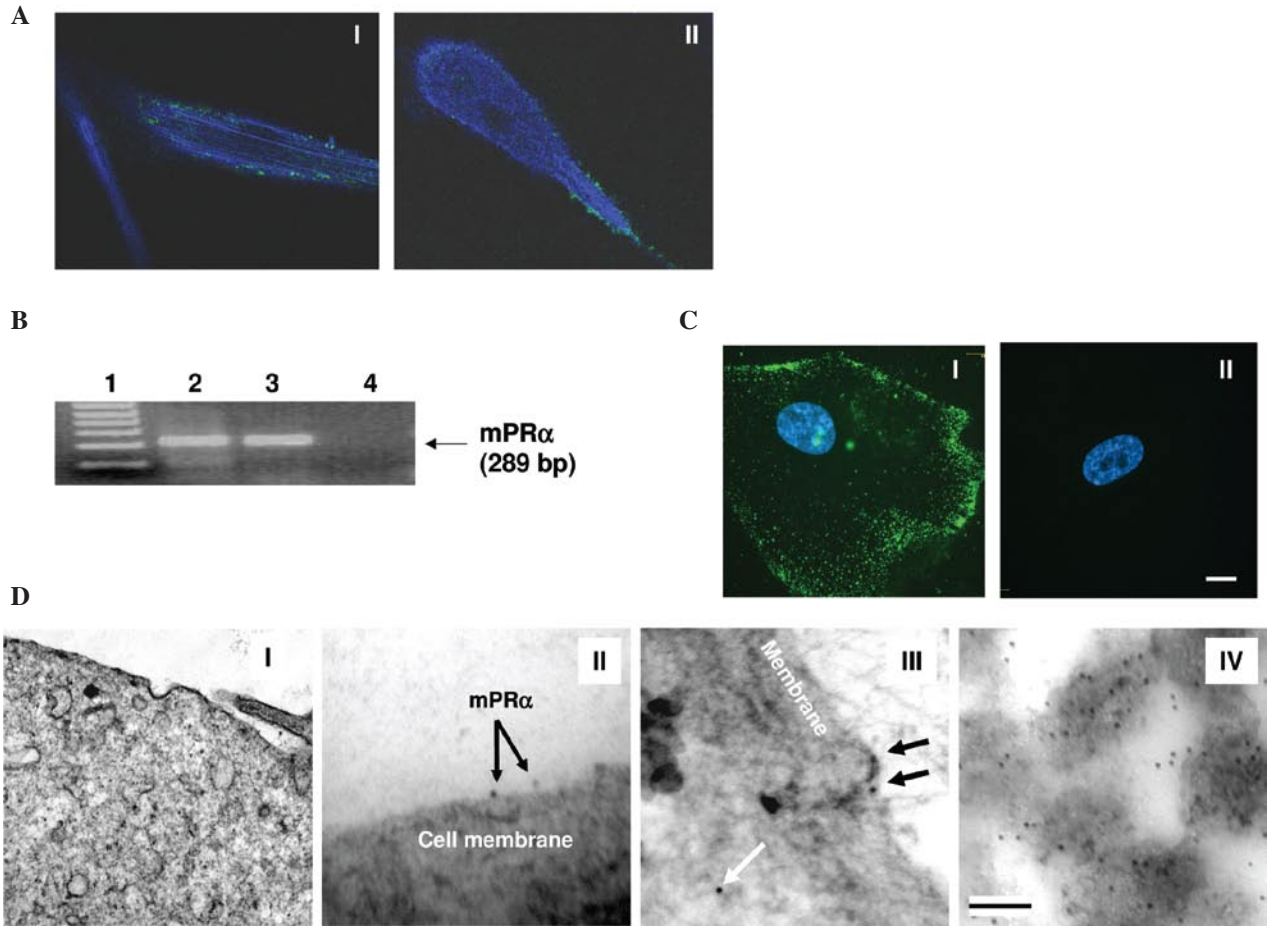


Figure 1. P4 binding, expression and cellular distribution of mPRα in M11 cells. (A) Confocal images of M11 cells incubated with P4 3-(O-carboxymethyl) oxime-BSA-FITC at room temperature for 20 min (I) and 45 min (II). Green represents progesterone and blue, phalloidin. (B) RT-PCR analysis for mPRα: Lane 1, DNA ladder; lane 2, cDNA from M11 cells; lane 3, cDNA from myometrial tissue; lane 4, negative control (no cDNA input). (C) Immunofluorescent analysis for mPRα in an M11 cell showing the distribution of mPRα (green) across the cell membrane. The nucleus was counterstained with DAPI (blue) (I); II is the negative control. Scale bar, 10 μm. (D) mPRα localisation and the cellular morphology of M11 cells using TEM. M11 cells had intact cytoplasmic machinery and a distinct cell membrane (I). Immunogold electron microscopy revealed mPRα localisation and distribution detected via a secondary antibody conjugated to 10-nm gold beads. This revealed specific staining on the cell membrane (indicated by black arrows, II and III) and within the cytoplasm (indicated by white arrow, III). IV is a control preparation depicting the size of the 10-nm gold beads conjugated to the anti-rabbit secondary antibody used in these preparations. Scale bar, 100 nm, with the exception of I, 250 nm.

inactivated FBS, penicillin and streptomycin. Cells were washed with 0.1 M Sorensen's phosphate buffer (pH 7.0) and fixed in 0.1% glutaraldehyde for 30 min at room temperature. Cells were washed and then blocked with 10% donkey serum for 30 min at room temperature. Anti-clathrin antibody (Sigma-Aldrich) diluted 1:40 with Sorensen's phosphate buffer containing 1% donkey serum and 0.1% sodium azide was applied to the cells and incubated overnight at 4˚C. Washed cells were incubated in 18-nm gold labelled donkey anti-goat IgG secondary antibody, 1% donkey serum and 0.1% sodium azide for 1 h at room temperature. Post-fixation was performed in 2% glutaraldehyde for 10 min followed by fixation with 1% osmium tetroxide for 1 h at room temperature. The cells were dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections were collected on copper grids and stained with uranyl acetate and lead citrate prior to observation by transmission electron microscopy (EM) using a JEOL 2000 FX Transmission Electron Microscope.

M11 cells were also probed with mPRα (hmPRα, 836-4) antibody and goat anti-rabbit IgG 10-nm gold labelled secondary antibody. These cells were treated using the aforementioned protocol with the following exceptions: firstly, cells were blocked in 1% bovine serum albunin (BSA), 10% goat serum; secondly, antibodies were diluted in 1% goat serum, 1% BSA and 0.1% sodium azide made up in 0.1 M Sorensen's phosphate buffer pH 7.0.

P4 binding. M11 cells incubated in Opti-MEM (Invitrogen) supplemented with 30 mM HEPES and 0.2% BSA were grown on glass bottomed Petri dishes. These were incubated with 100 nM progesterone 3-(O-carboxymethyl)oxime: BSA-fluorescein isothiocynate conjugate (Sigma-Aldrich) for 10 and 45 min at room temperature prior to fixation with 4% paraformaldehyde for 10 min. After subsequent washes with PBS, the cells were permeabilised with 0.1% saponin, 0.5% BSA in PBS for 20 min. Fixed cells were then washed with PBS and incubated with a 1:300 dilution of phalloidin-Alexa fluor 568 (Molecular probes, UK) in 0.5% BSA PBS for 15 min prior to further PBS washes. The M11 cells were mounted with antifade solution (Citifluor-PBS, Citifluor Ltd., UK) and visualised using a Nikon Eclipse TE2000S confocal microscope.

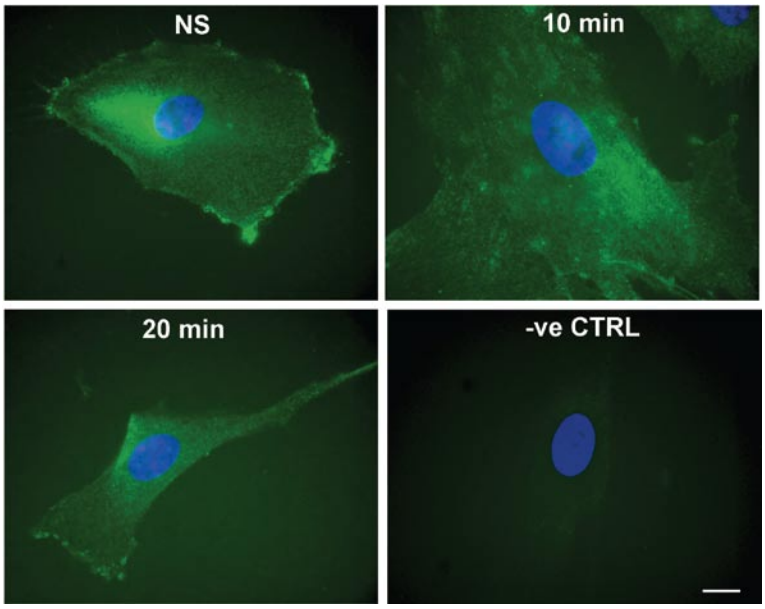
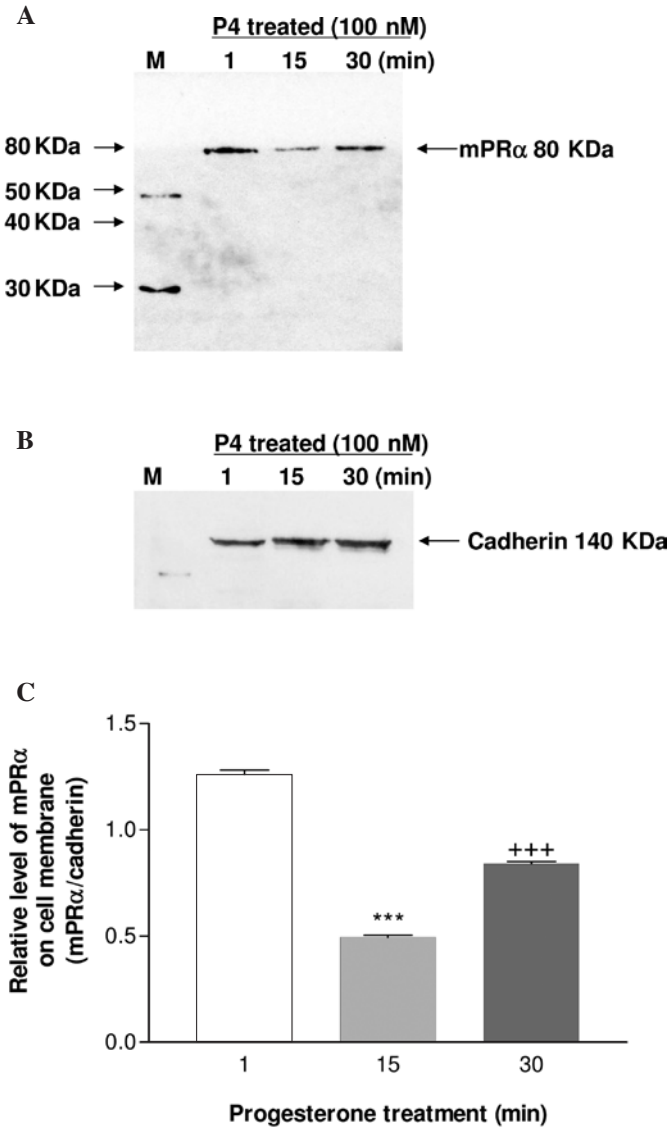


Figure 2. Distribution of mPRα in M11 cells upon stimulation with P4. Under basal conditions (NS), mPRα was predominantly confined to the cell surface. After 10 min of treatment with 100 nM P4, translocation of mPRα from the cell surface towards the cytoplasm was evident. However, after exposure to P4 for 20 min, a substantial amount of mPRα protein was localised back at the plasma membrane. Scale bar, 10 μm. -ve CTRL, negative control immunostaining with secondary antibody only.



Statistical analysis. Expression mPRα protein was determined using Western blot analysis as described previously (11) and with semi-quantitative densitometry performed using NIH ImageJ (v1.26).

Results

Membrane binding of progesterone, expression and cellular distribution of mPRα in M11 cells. Membrane progesterone sites were detected in M11 cells upon incubation with P4 3-(O-carboxymethyl)oxime-BSA-FITC at room temperature for 20 min (Fig. 1A, panel I) and 45 min (Fig. 1A, panel II).

Expression of mPRα at the gene level was confirmed in M11 cells via RT-PCR (Fig. 1B). Furthermore, mPRα expression was verified at the protein level through indirect immunofluorescence (Fig. 1C) and immunogold electron microscopy (Fig. 1D), both of which depict characteristic plasma membrane localisation in M11 cells under basal conditions.

Electron microscopy is the gold standard and the most robust technique for determining the cellular distribution of mPRα within M11 cells. Using this technique, we verified that M11 cells have an intracellular environment rich in the mandatory organelles essential for transcription and translation processes (Fig. 1D, panel I). Immunogold EM provided

Figure 3. Western blot analysis of mPRα protein M11 plasma membranes after exposure to P4 for a 1-, 15- and 30-min duration. (A) M11 plasma membrane preparations (P4 100 nM for 1, 15 and 30 min) probed with an mPRα antibody resulting in 80 kDa bands. (B) Immunoblotting with a cadherin antibody shows equal membrane protein loading on the gel for each time point. (C) Semi-quantification of mPRα protein on M11 plasma membrane samples by the normalisation of mPRα protein with cadherin protein and semi-quantitative densitometry performed using NIH ImageJ (v1.26). Statistical differences were determined for each of the different P4 exposure times (***P<0.001 compared with 1 min; $^{+++}$P<0.001 compared with 1 and 15 min; one way ANOVA).

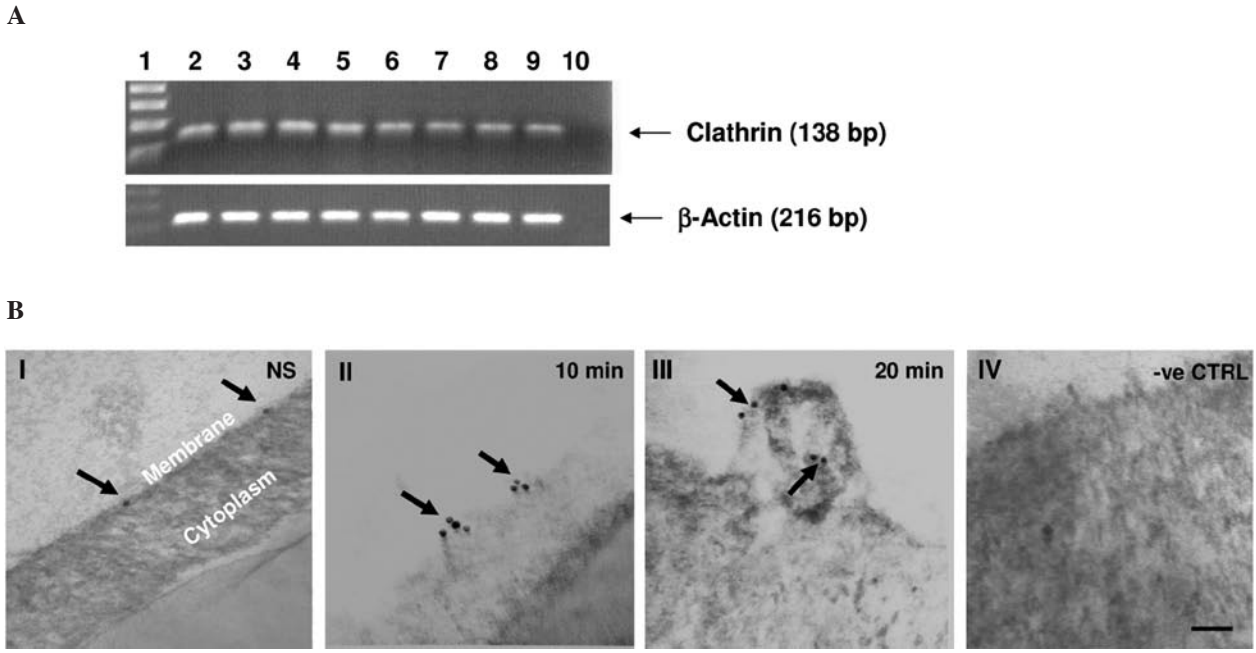


Figure 4. Expression of clathrin in M11 cells and its cellular distribution. (A) RT-PCR analysis for clathrin and β-actin in P4-treated M11 samples. Lane 1, DNA ladder; lane 2, NS; lanes 3-9, cDNA from M11 cells treated with P4 for 1, 3, 5, 10, 15, 20 and 30 min respectively; lane 10, negative control. (B) Immunogold electron microscopy illustrating the localisation and distribution of clathrin in M11 cells detected via a secondary antibody conjugated to 18 nm gold beads. This revealed clathrin (arrows) to be dispersed in non-P4-treated M11 cells (I). After exposure to P4 for 10 min, clathrin appeared to form clusters (II). At 20 min of P4 treatment, clathrin was present both on the cell membrane and internalised within the cytoplasm (III). IV, negative control. Scale bar, 100 nm.

irrefutable evidence for the first time that mPRα occupies a location at the cell membrane in M11 cells under basal conditions (Fig. 1D, panel II). Nevertheless, mPRα also occasionally occupied an intracellular locality within the cell cytoplasm (Fig. 1D, panel III).

Sequestration and trafficking of mPRα from the plasma membrane after P4 stimulation. To study the potential trafficking of mPRα and to further determine its distribution pattern, immunofluorescent analyses were conducted in M11 cells treated with P4 (100 nM) for 5, 10 and 20 min. In untreated cells, mPRα was localised both at the cell surface and cytoplasm (Fig. 2; NS). Following 5 min of P4 treatment, there was an altered distribution of the receptor with mPRα present both on the cell membrane and in the cytoplasm (data not shown). At 10 min of treatment, there was a clear decline in mPRα localised to the cell membrane (Fig. 2; 10 min), whereas at 20 min the receptor was again detected on the plasma membrane (Fig. 2; 20 min).

To confirm trafficking of mPRα following progesterone treatment, solubilised M11 plasma membrane preparations treated with 100 nM P4 for 1, 15 and 30 min (Fig. 3A) were subjected to Western blot analysis with mPRα specific antibodies. Plasma membrane levels of mPRα protein varied with the duration of P4 exposure. The highest level of mPRα protein was present at the membrane after a 1-min exposure to P4, with a subsequent reduction in levels at 15 min of treatment and an increase at 30 min. Western blot analysis using an antibody directed against cadherin (Fig. 3B), a transmembrane protein involved in cell adhesion, was used as a control to normalise mPRα protein levels in the plasma membrane (Fig. 3B). Normalised mPRα/cadherin levels showed a significant difference ($P<0.001$) when comparing protein from the 15-min P4 exposure to the 1-min exposure, with a reduction in mPRα present at the plasma membrane at 15 min. A significant difference ($P<0.001$) in mPRα protein levels also occurred when comparing the 30-min P4 exposure to the 1- and 15-min P4 exposures, with 30-min P4 levels being statistically lower than the 1-min levels, but higher than the 15-min levels (Fig. 3C). These data support the hypothesis that mPRα is expressed on the cell membrane, rapidly internalised after stimulation with P4, and then recycled/shuttled back to the plasma membrane.

Expression and distribution of clathrin in M11 cells. As mentioned previously, a major mechanism for the internalisation of GPCRs is via clathrin-mediated endocytosis. Therefore, in order to investigate the potential involvement of clathrin in the endocytosis of mPRα, we characterised its expression and trafficking in M11 cells. Semi-quantitative RT-PCR analysis revealed that, firstly, clathrin mRNA is expressed in M11 cells, and, secondly, that its expression remains unaltered with up to 30 min of P4 treatment (Fig. 4A). Immunogold EM was also used to reveal that there are distinct changes in the distribution of clathrin in P4-stimulated M11 cells. After 10 min of P4 treatment, there was a change in clathrin distribution, with a noticeable increase in cluster sequestration (Fig. 4B, panel II; 10 min compared to panel I; NS). At 20 min of P4 treatment, there was a distinct internalisation of clathrin in the cytoplasm, with some remaining on the plasma membrane (Fig. 4B, panel III; 20 min).

Co-distribution of mPRα with clathrin. Under basal conditions, clathrin was distributed throughout the cell (Fig. 5A; NS), whereas mPRα was predominantly limited to the plasma membrane. Little colocalisation between clathrin and mPRα

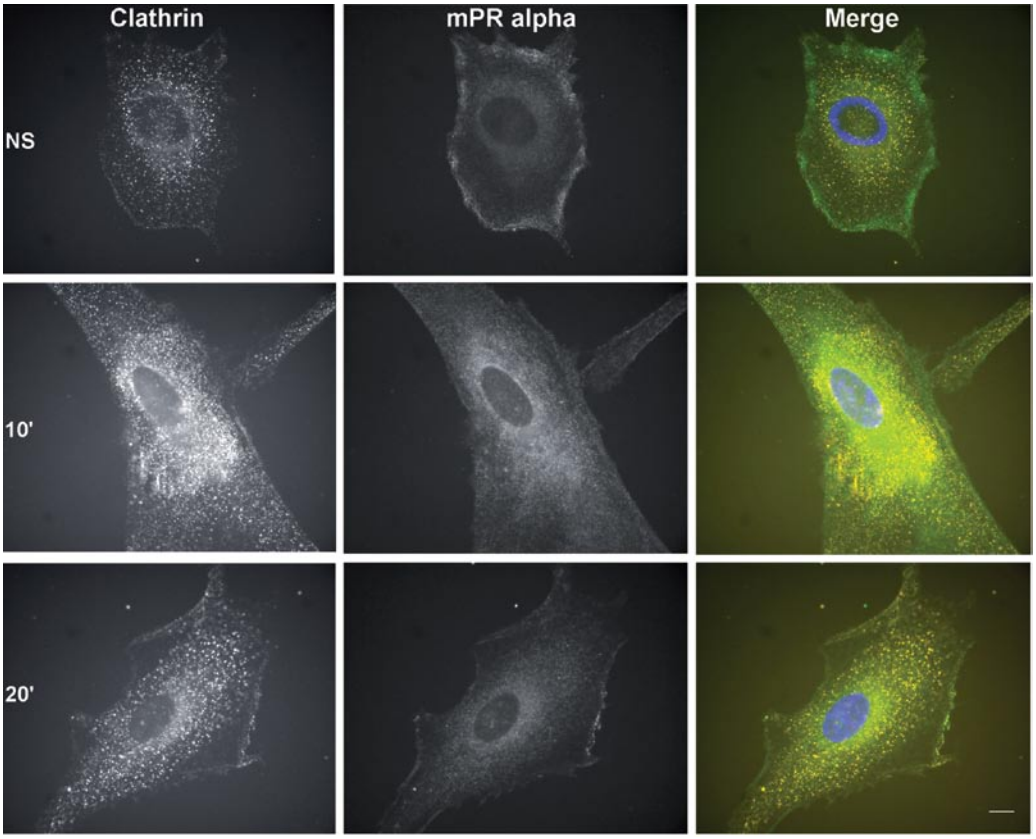


Figure 5. Colocalisation of mPRα and clathrin in P4-treated M11 cells. The immunofluorescent images depict the distribution and partial colocalisation of mPRα (green) and clathrin (orange) in M11 cells. Nuclei were counterstained with DAPI (blue). Clathrin and mPRα colocalisation was determined in M11 cells after treatment with P4 for 10 and 20 min. Scale bar, 10 μM.

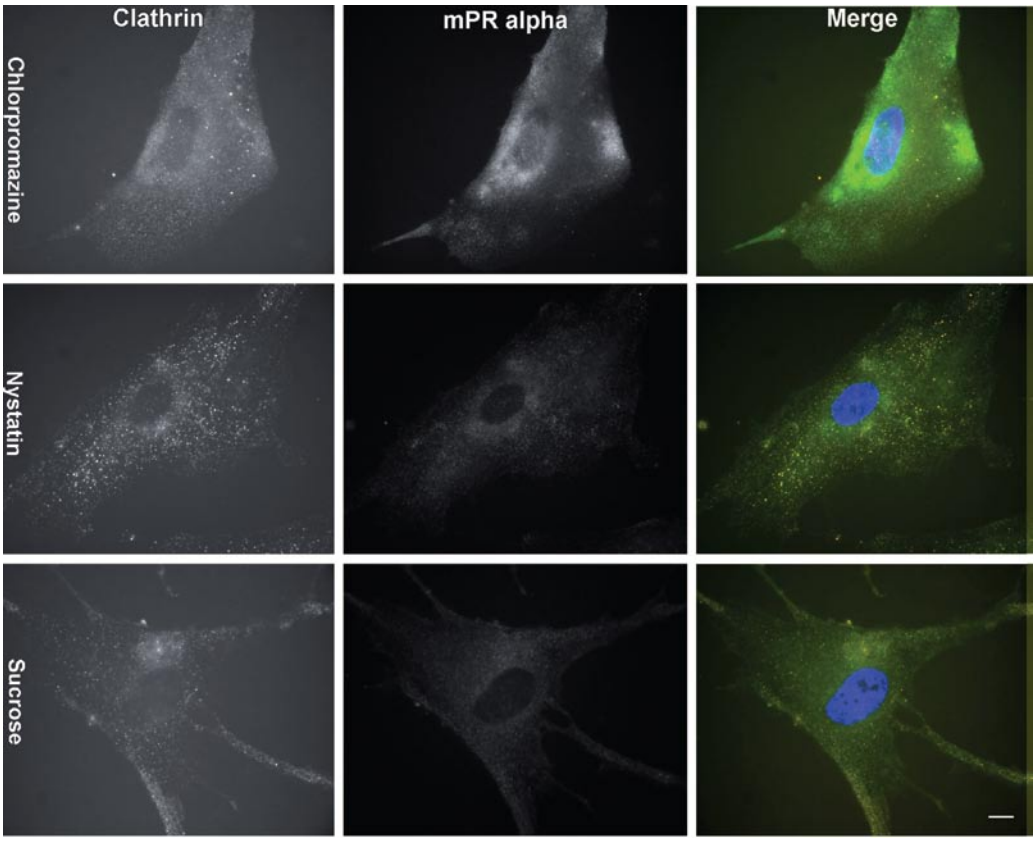


Figure 6. Effect of inhibitors on clathrin and mPRα endocytosis in M11 cells. M11 cells were exposed to P4 for 10 min prior to treatment with specific inhibitors to determine how this influences clathrin and mPRα endocytosis. The immunofluorescent images depict clathrin (orange) and mPRα (green) treated with chlorpromazine, nystatin or sucrose. Scale bar, 10 μM.

was evident (Fig. 5A; NS). At 10 min of P4 exposure, both clathrin and mPRα were increasingly localised within the cell (Fig. 5A; 10 min). Partial colocalisation between mPRα and clathrin was determined, visible in the merged image as orange-coloured speckles (Fig. 5A; 10 min). The distribution of both mPRα and clathrin at 20 min of P4 treatment seemingly reverted to that observed under basal conditions (Fig. 5A; 20 min).

Use of endocytotic inhibitors to investigate potential internalisation pathways of mPRα. At 10 min of P4 exposure, internalisation and partial colocalisation of mPRα and clathrin was evident in the M11 cells (Fig. 5A; 10 min). Inhibitors of endocytosis were used on M11 cells, which were then exposed to 10 min of P4 treatment to examine the resultant distribution and colocalisation properties of clathrin and mPRα. Treatment of M11 cells with chlorpromazine resulted in an atypical clathrin distribution, with a reduction in observed clathrin speckles (Fig. 6; chlorpromazine). mPRα appeared to be sequestered in much larger, but fewer, clusters localised at either the cell membrane or the cellular interior (Fig. 6; chlorpromazine). No clear colocalisation between clathrin and mPRα was apparent (Fig. 6; chlorpromazine). Nystatin inhibitor did not alter the clathrin or mPRα distribution, nor their partial colocalisation, which is readily observed under standard conditions (Fig. 6; nystatin). Sucrose treatment of M11 cells altered mPRα distribution (Fig. 6; sucrose). The internal clustering of mPRα typically seen in M11 cells was also less apparent, with no observable internal sequestration of mPRα (Fig. 6; sucrose). Clathrin and mPRα did not appear to colocalise when treated with sucrose.

Discussion

In this study, we investigated the cellular distribution and trafficking of mPRα in a human myometrial cell line (M11). We chose this experimental model since it has been shown to have morphological characteristics typical of myometrial cells (16), and also expresses mPRα (11,17).

Immunofluorescent analysis revealed that mPRα has a similar subcellular distribution to that previously observed in human myometrial cells under basal conditions (11), and is localised on the plasma membrane, with some cytoplasmic expression. In agreement with these results, membrane localisation of mPRs has been demonstrated by several research groups in a variety of cell types (1,10,18-22). Other researchers have reported mPRs to be localised intracellularlly in the endoplasmic reticulum (23-25). EM is the gold standard in high resolution imaging, and was used to conclusively determine the sub-cellular distribution of mPRα. mPRα occupies a cell membrane location under basal conditions, but intracellular mPRα is also apparent. These novel data incorporated EM for the first time to address mPRα distribution in any given cell type.

An important characteristic of 7TMRs is that they are rapidly internalised upon activation in a ligand-dependent manner, an event critical for their proper functioning (26). Currently, there is no information on the trafficking of mPRα. Therefore, we utilised an immunofluorescent approach to investigate the trafficking of mPRα. mPRα protein was redistributed from the cell membrane in unstimulated cells, becoming internalised at 10 min of P4 exposure. mPRα was presented back on the cell membrane at 20 min, but not to the levels previously seen under basal conditions. This was ligand specific, since treatment with the unrelated steroid cortisol (100 nM) and the nuclear PR activator R5020 (30 nM) did not induce any significant internalisation of the receptor in M11 cells (data not shown). The mPRα internalisation was confirmed by specific single-point P4 binding studies. At 20 min, there was a significant decrease in [^{3}H]-P4 binding on the plasma membrane of M11 cells as compared with 1-min P4 treatment (data not shown). The decrease in membrane-bound mPRα may act as a desensitisation process to modulate rapid P4 signalling responses.

These data are consistent with the data in the literature, which indicate that the intracellular segregation of receptors due to endocytotic events is a common fate for 7TMRs. This provides another explanation for why several 7TMRs appear to be expressed in the cytoplasm (27), and may also explain why some investigators have observed mPRs intracellularly. In kidney tissue, mPRα was found to be restricted to the endoplasmic reticulum (ER) due to a C-terminal ER retention motif (25). This is not unusual for 7TMRs, since many require accessory proteins to ensure transport to the plasma membrane (28,29). An example is the melanocortin 2 receptor, which is localised within the ER and cannot be transported to the plasma membrane unless it is co-expressed with the melanocortin 2 receptor accessory protein (28). It possible, therefore, that mPRα requires an accessory protein that is expressed in tissue- or cell-specific manner, thus affecting its transport to the membrane. These studies highlight the difficulty in drawing any firm conclusions on 7TMR trafficking (30), and present an arguement for a higher order of complexity regarding mPRα distribution.

The next step of this study was to further dissect the endocytotic pathway(s) involved in mPRα trafficking. Endocytosis of activated receptors can be mediated via caveolae/lipid raft mechanisms (32) and clathrin-dependent endocytosis. Lipid rafts are sterol- and sphingolipid-rich membrane domains comprising caveolae subdomains involved in the endocytosis of various ligands (32,33). However, for most 7TMRs, endocytosis is implemented via clathrin-mediated mechanisms involving the formation of coated pits at the cell surface that concentrate surface protein/cargo for internalisation (34).

The role of clathrin in the endocytosis of mPRα in M11 cells was investigated. Following progesterone treatment, there was partial co-localisation of clathrin with mPRα, evident in Fig. 5 as orange speckles. In addition, co-immunoprecipitation studies show that clathrin is immunoprecipitated with mPRα, indicating a close association between these two proteins and suggesting that they may be coupled under certain conditions (data not shown). M11 cells were treated with various inhibitors of endocytosis prior to progesterone treatment. Inhibitors were chosen according to their ability to discriminate and inhibit different types of endocytotic pathways. Treatments included chlorpromazine and sucrose inhibitors specific for clathrin-mediated internalisation and nystatin, which inhibits caveolae/lipid-raft mediated endocytosis (35,36). These inhibitors do not demonstrate absolute selectivity between endocytotic pathways; nevertheless, they are commonly used to differentiate trafficking routes (37). Chlorpromazine inhibitor acts by preventing the adapter protein AP2 from binding to clathrin-

coated pits (12,38), whereas sucrose inhibitor acts by interfering with clathrin recruitment, coated pit formation and endocytosis (39). These inhibitors prevented the clathrin-mediated endocytosis of mPRα. Nystatin is a polyene antibiotic that inhibits caveolae/lipid raft-mediated endocytosis by the sequestration of cholesterol within the membrane, but does not affect clathrin-mediated endocytosis. Nystatin pre-treatment did not alter the internalisation of mPRα after 10 min of P4 exposure. Collectively, these data do not support a role for caveolae in the mediation of mPRα endocytosis. In line with these observations, 7TMRs have been found to predominantly use the clathrin-dependent endocytotic pathway, whereas caveolin and clathrin/caveolin-independent pathways appear to be used to a lesser extent (8,40,41). Interestingly, other uncharacterised endocytotic pathways may be involved in receptor trafficking. For instance, corticotropin-releasing hormone receptors appear to undergo endocytosis independent of clathrin or caveolin upon activation by urocortin (42). It is therefore possible that mPRα is also capable of internalising via another route, independent of clathrin or caveolin.

Potential changes in the phosphorylation status of the receptor may also affect its trafficking dynamics. There are two different types of kinases that can phosphorylate GPCRs: i) second-messenger-dependent kinases (e.g., PKC), and ii) GPCR kinases (GRKs). β-arrestins have an essential role following receptor phosphorylation, functioning as adaptor molecules that link receptors to clathrin-coated pits (43-45). Potential phosphorylation sites of mPRα were predicted using the NetPhosK Server. mPRα can be phosphorylated at two serine sites (positions 219, 229), three threonine sites (positions 32, 57 and 232) and three tyrosine sites (positions 143, 194 and 304). Future studies, using mutational analysis of these sites, will provide better insight regarding the potential role of mPRα phosphorylation in endocytotic events.

In summary, these data provide the first definitive evidence that mPRα can be localised on the plasma membrane and within the cytoplasm. This is particularly important given that receptor number on the cell surface is a fine balance between the rate of internalisation, recycling and new synthesis, and the fact that many receptors may also signal from endosomal compartments (3). Potential mechanisms involved in P4-mediated endocytosis have also been investigated, providing novel insight on mPR-mediated responses. Future studies should focus on determining mPRα degradation, recycling to the cell surface and activation of distinct intracellular pathways while internalised.

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