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RESEARCH PAPER

Arrestins differentially regulate histamine- and oxytocin-evoked phospholipase C and mitogen-activated protein kinase signalling in myometrial cells

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BACKGROUND AND PURPOSE

The uterotonins oxytocin and histamine, mediate contractile signals through specific G protein-coupled receptors, a process which is tightly controlled during gestation to prevent preterm labour. We previously identified G protein-coupled receptor kinase (GRK)2 and GRK6 as respective cardinal negative regulators of histamine H_1 and oxytocin receptor signalling. GRK-mediated phosphorylation promotes arrestin recruitment, not only desensitizing receptors but activating an increasing number of diverse signalling pathways. Here we investigate potential roles that arrestins play in the regulation of myometrial oxytocin/histamine H_1 receptor signalling.

EXPERIMENTAL APPROACH

Endogenous arrestins2 and 3 were specifically depleted using RNA-interference in a human myometrial cell line and the consequences of this for G protein-coupled receptor-mediated signalling were assessed using Ca^{2+}/inositol 1,4,5-trisphophate imaging and standard mitogen-activated protein kinase (MAPK) assays.

KEY RESULTS

Depletion of arrestin3, but not arrestin2 enhanced and prolonged H_1 receptor-stimulated Ca^{2+} responses, whilst depletion of either arrestin increased oxytocin receptor responses. Arrestin3 depletion decreased H_1 receptor desensitization, whilst removal of either arrestin isoform was equally effective in preventing oxytocin receptor desensitization. Following arrestin3 depletion oxytocin-induced phospho-extracellular signal-regulated kinase1/2 signals were diminished and histamine-stimulated signals virtually absent, whereas depletion of arrestin2 augmented extracellular signal-regulated kinase1/2 responses to each agonist. Conversely, depletion of arrestin3 enhanced p38 signals to each agonist, whilst arrestin2 suppression increased oxytocin-, but not histamine-induced p38 MAPK responses.

CONCLUSIONS AND IMPLICATIONS

Arrestin proteins are key regulators of H_1 and oxytocin receptor desensitization, and play integral roles mediating uterotonin-stimulated MAPK-signalling. These data provide insights into the *in situ* regulation of these receptor subtypes and may inform pathophysiological functioning in preterm labour.

Abbreviations

$[Ca^{2+}]_i$, intracellular calcium concentration; eGFP-PH, e-GFP-tagged pleckstrin homology domain of phospholipase Cδ; ERK, extracellular signal-regulated kinase; Go6976, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; MAPK, mitogen-activated protein kinase; pERK, threonine- and tyrosine-phosphorylated extracellular signal-regulated kinase

Introduction

Myometrial tone depends upon the dynamic balance between contractile [e.g. voltage-dependent and -independent Ca^{2+} channel activity, phospholipase C (PLC)-coupled receptor activity (Sanborn, 2007)], and relaxatory inputs [e.g. plasmalemmal and sacroplasmic reticulum Ca^{2+} ATPases, and Na^+/ Ca^{2+} exchangers, cyclic AMP concentrations and K^+ channel activity (Sanborn, 2000; 2007; Yuan and Lopez Bernal, 2007)]. One important excitatory input is provided by the wide variety of PLC-coupled receptors (e.g. oxytocin, prostaglandin $F_{2\alpha}$, histamine H_1) expressed on human myometrial cells, the activation of which stimulates the production of inositol 1,4,5-trisphosphate and diacylglycerol to initiate and prolong smooth muscle contraction through activation of Ca^{2+} mobilization and influx pathways respectively (Ku *et al.*, 1995; Holda *et al.*, 1996; Sanborn, 2001). During pregnancy the uterus is subjected to extensive remodelling and stretch due to the developing fetus. Despite these physical changes, it is essential that the uterine environment is maintained in a quiescent state throughout gestation to prevent preterm labour. At present, the exact mechanisms by which this process is achieved are not fully understood, but short-term [receptor desensitization and/or down-regulation (Engelhardt *et al.*, 1997; Phaneuf *et al.*, 1997; Willets *et al.*, 2008; 2009)] as well as longer-term hormonal regulation of receptor expression levels are likely to be involved (Fuchs *et al.*, 1984; Matsumoto *et al.*, 1997; Helmer *et al.*, 1998; Brodt-Eppley and Myatt, 1999).

Most G protein-coupled receptors are tightly regulated through the action of G protein-coupled receptor kinases (GRK) to prevent prolonged or inappropriate signalling (Willets *et al.*, 2003). GRK proteins phosphorylate agonist-occupied G protein-coupled receptors (GPCRs) at key serine or threonine residues within the third intracellular loop or *C*-terminal tail, a process that usually leads to the recruitment of non-visual arrestin proteins (Willets *et al.*, 2003; DeWire *et al.*, 2007). Arrestin binding physically prevents GPCR/G protein interactions, whilst also promoting GPCR desensitization and internalization. Interestingly, accumulating evidence highlights that arrestin proteins have many more diverse roles than mediation of receptor desensitization and internalization (Luttrell and Lefkowitz, 2002), including acting as agonist-regulated adaptor scaffolds for extracellular signal-regulated kinases (ERK) and other mitogen-activated protein kinases (MAPK) (Luttrell and Lefkowitz, 2002; DeWire *et al.*, 2007; Gesty-Palmer and Luttrell, 2008). We recently identified regulatory roles for GRK2 in H_1 (Willets *et al.*, 2008) and GRK6 in oxytocin receptor (Willets *et al.*, 2009) signalling, respectively, in both ULTR (an immortalized human myometrial cell line) and primary myometrial cells. Because GRK-mediated GPCR phosphorylation promotes arrestin association, we have now gone on to investigate the potential roles of arrestin proteins in H_1 and oxytocin receptor desensitization, as well as the ability of arrestins to affect receptor coupling to MAPK pathways.

Although little is presently known about the potential roles of arrestins in H_1 receptor signalling, studies utilizing model cell systems recombinantly overexpressing either GPCR and/or arrestin constructs, highlight a potential role for arrestins in the regulation of oxytocin receptor signalling (Oakley *et al.*, 2001; Smith *et al.*, 2006). However, it should be noted that such overexpression studies are often not reflective of how GPCRs are regulated within their native environment (Tobin *et al.*, 2008). Indeed, accumulating evidence indicates that the regulation of GPCRs is likely to be highly dependent on the cell background (Kong *et al.*, 1994; Simon *et al.*, 2003; Smith *et al.*, 2006; Willets *et al.*, 2009). Therefore, to assess the involvement of arrestins in different aspects of H_1 and oxytocin receptor signalling/regulation, we have utilized small interfering (si)RNAs to specifically ablate endogenous arrestin2 and arrestin3 expression in the ULTR human myometrial cell line.

Methods

All drug and molecular target nomenclature conforms to the British Journal of Pharmacology's Guide to Receptors and Channels (Alexander *et al.*, 2009).

Cell culture

An immortalized human myometrial cell line (ULTR) (Perez-Reyes *et al.*, 1992) previously extensively characterized by us (Brighton *et al.*, 2009; Willets *et al.*, 2008; 2009) and others (Olson *et al.*, 2003; Ball *et al.*, 2006), were maintained in Dulbecco's minimal essential medium, supplemented with 10% fetal calf serum, penicillin (100 $IU \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$), and amphotericin B (2.5 $\mu g \cdot mL^{-1}$) and Glutamax-1. Cells were maintained under humidified conditions at 37°C, in air/5% CO_2.

siRNA-targeted arrestin depletion

To deplete endogenous arrestin expression, cells were transfected with specific siRNAs designed to target either human arrestin2 (ARRB1) (5′-GGAGAUCUAUUACCAUGGtt-3′) or arrestin3 (ARRB2) siRNA (5′-CGAACAAGAUGACCAGGUAtt-3′). ULTR cells were seeded 24 h before transfection at a density of 150 000 cells per well of a six-well culture plate, and transfected with various concentrations (10 or 100 nM) of either negative control (non-targeting), anti-arrestin2, anti-arrestin3 or a combination of anti-arrestin2 and anti-

arrestin3 siRNAs. After 48 h, cells were lysed and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separation and transferred to nitrocellulose as described previously (Willets *et al.*, 2001). Arrestin expression was detected using the A1CT polyclonal antibody (Ahn *et al.*, 2004b) raised against arrestin2, which cross-reacts with arrestin3 allowing visualization of both proteins. The relative expression of individual arrestin proteins was determined using the GeneGnome image analysis system and software (Syngene, Cambridge, UK).

Assessment of oxytocin- and histamine-stimulated Ca^{2+} signalling

To assess oxytocin- and histamine-mediated intracellular calcium concentration ($[Ca^{2+}]_i$) changes, ULTR cells were seeded into 96-well plates and grown to 90% confluency. Cells were washed with Krebs buffer [composition (mM); HEPES 10, $NaHCO_3$ 1.3, D-Glucose 11.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, KCl 4.7, NaCl 118 and $CaCl_2$ 1.3, pH 7.4] and loaded with 3 μM fluo4-AM (room temperature, 1 h). Cell monolayers were washed again with Krebs buffer prior to challenge with increasing concentrations of either oxytocin or histamine for various time periods. Agonist-stimulated fluorescence intensity changes were measured using a NovoStar imaging system (BMG Labtech, Aylesbury, UK), and plotted graphically as the maximum increase in fluorescence minus basal fluorescence. To assess whether arrestin depletion affected oxytocin- or histamine-stimulated Ca^{2+} signalling, ULTR cells were seeded 24 h before transfection at a density of 10 000 cells per well and transfected with either negative control (100 nM), anti-human arrestin2 (100 nM) or anti-human arrestin3 (10 nM) siRNAs. Transfection was achieved using Interferin™ transfection reagent (Polyplus, New York, NY, USA), as per manufacturers' instructions.

Determination of oxytocin- and histamine-stimulated phosphoinositide turnover and receptor desensitization in single cells

Oxytocin- and H_1 receptor-stimulated PLC activity was assessed in 'real time' using the e-GFP-tagged pleckstrin homology domain of phospholipase Cδ (eGFP-PH) as a biosensor of receptor-mediated PLC activity (Willets *et al.*, 2005; 2008; 2009). Cells were seeded onto glass coverslips and transfected with eGFP-PH (0.5 μg) using Lipofectamine2000 (Invitrogen, Paisley, UK) as per manufacturers' instructions. After 48 h, agonist-stimulated fluorescent eGFP-PH membrane-cytoplasmic translocations were assessed using an Olympus FV500 scanning laser confocal microscope as described previously (Willets *et al.*, 2005; 2008). Cells were maintained at 37°C using a temperature controller and micro-incubator (PDMI-2 and TC202A; Burleigh, Digitimer, Cambridge, UK), and perfused with Krebs–Henseleit buffer (composition: NaCl 134 mM, KCl 6 mM, $MgCl_2$ 1 mM, glucose 10 mM, HEPES 10 mM and $CaCl_2$ 1.3 mM, pH 7.4) at 5 $mL \cdot min^{-1}$. Images were captured using an oil immersion 60× objective, with inositol 1,4,5-trisphosphate levels determined by increases in cytosolic fluorescence in a defined area of interest exactly as described previously (Willets *et al.*, 2005; 2008). Oxytocin and H_1 receptor desensitization was determined using our previously validated protocols. Briefly, to assess oxytocin receptor desensitization, cells were stimulated with a maximal oxytocin concentration (100 nM) for 30 s (termed R1), followed by a 5 min wash period before a second 30 s oxytocin challenge (100 nM, termed R2). R2 responses were significantly attenuated compared with R1 and the resulting reduction in the R2/R1 ratio is interpreted as an indication of oxytocin receptor desensitization (Willets *et al.*, 2009). Due to the presence of a significant receptor reserve, a slightly different protocol was applied to observe H_1 receptor desensitization (Willets *et al.*, 2008). Here, cells were challenged with an approximate EC_{50} histamine concentration (10 μM) for 30 s, 5 min before (R1) and 5 min after (R2) a desensitizing histamine pulse (100 μM, for 1 min). Again reduced R2/R1 ratios were interpreted as an indication of receptor desensitization (Willets *et al.*, 2008).

Detection of MAPK activation

Agonist-driven ERK1/2 (MAPK3/MAPK1) activity was detected using Western blotting techniques as described previously (Brighton *et al.*, 2009). Agonist-stimulated p38 MAPK (MAPK11-14) phosphorylation was also detected through a similar Western blotting approach. Briefly, ULTR cells were seeded into six-well plates and grown to confluency. Cells were then deprived of serum for 24 h before agonist addition. Next, signalling was terminated by the addition of lysis buffer [composition: 20 mM Tris-HCl (pH 7.4), 1% ($v \cdot v^{-1}$) Triton X-100, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanidate, 500 μM phenylmethanesulphonylfluoride, 0.1 $mg \cdot mL^{-1}$ leupeptin, 0.2 $mg \cdot mL^{-1}$ benzamidine, and 0.1 $mg \cdot mL^{-1}$ pepstatin]. Insoluble material was cleared by centrifugation and an equal volume of 2× sample buffer [composition: 250 mM Tris-HCl, pH 6.8, 0.01% ($w \cdot v^{-1}$) bromophenol blue, 2% ($w \cdot v^{-1}$) sodium dodecyl sulphate, 40% ($v \cdot v^{-1}$) glycerol and 50 mM ditiothreitol] and added before heating (5 min, 100°C) and gel loading. Samples were separated by SDS-PAGE and transferred to nitrocellulose using Western blotting techniques. Detection of threonine- and tyrosine-phosphorylated extracellular signal-regulated kinase (pERK)1/2 was via a specific anti-pERK1/2 antibody (Promega, Southampton, UK), which detects the dual phosphorylated P-loop pTEpY motif of ERK1 and ERK2; phospho-p38 MAPK (pTGpY) was detected using a specific anti-phospho-p38 antibody (Cell Signaling, Madison, WI, USA). Immune-reactive bands were visualized using HRP-conjugated anti-rabbit secondary antibody (Sigma, Poole, UK), ECL reagent and Hyperfilm (GE Healthcare, Little Chalfont, UK). Densitometric analysis of the resultant autoradiographs was undertaken using the GeneGnome image analysis system and software (Syngene, Cambridge, UK). For data relating to ERK1/2, the densities of p42 and p44 ERK proteins (ERK 1 and 2) were averaged and related to basal (unstimulated) levels. To ensure that all samples contained the same levels of protein, total ERK and p38 levels were determined by running additional gels in parallel with the detection of pERK and phospho-p38. For ERK1/2 samples, uniform protein loading was confirmed by detection of total-ERK1/2 proteins using an anti-total ERK1/2 antibody (Santa Cruz, CA, USA) and for p38 samples, an antibody against total p38 (Cell Signaling, Madison, WI, USA).

Data analysis

All concentration–response curves were generated and EC_{50} values determined using non-linear regression analysis software Prism, version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Data were analysed using one-way or two-way ANOVA, followed by appropriate *post hoc* testing (Excel 5.0, Microsoft, Redmond, WA, USA). Significance was accepted when $P < 0.05$.

Results

Oxytocin- and histamine-mediated elevation of $[Ca^{2+}]_i$

Stimulation of fluo-4-loaded cells with either oxytocin or histamine resulted in concentration-dependent increases in fluorescence indicating elevation of $[Ca^{2+}]_i$ with typical peak and plateau profiles (Figure 1A). Concentration–response analysis revealed EC_{50} values of 1.1 nM [pEC_{50} (M) = 8.95 ± 0.20] and 105 nM [pEC_{50} (M) = 6.98 ± 0.16] for oxytocin and histamine respectively (Figure 1B).

Depletion of arrestin isoform expression in ULTR cells

To optimize endogenous arrestin protein depletion, ULTR cells were transfected with either 10 or 100 nM of siRNA targeting arrestin2, arrestin3 or a negative control siRNA. Initial experiments revealed that maximal arrestin depletion could be achieved 48 h after transfection (data not shown). Optimal arrestin2 depletion was attained following application of 100 nM anti-arrestin2 siRNA (Figure 2A and C). The A1CT antibody also detects arrestin3, albeit with lower affinity, enabling visualization of both arrestins on one blot. Increased exposure of the same blot (Figure 2B and C) highlighted the successful and selective depletion of arrestin3 immunoreactivity with concentrations of anti-arrestin3 siRNA of >10 nM. We routinely observed a >70% reduction in the expression of the targeted arrestin isoform when compared with cell lysates transfected with negative control siRNA. Importantly, each anti-arrestin siRNA appeared selective for the isoform targeted (Figure 2A–C). In all subsequent experiments 100 nM of anti-arrestin2 and 10 nM of anti-arrestin3 siRNA were used to maximally deplete targeted endogenous arrestin isoform.

Arrestin depletion enhances oxytocin- and histamine-stimulated Ca^{2+} signals

To assess whether arrestin depletion affected oxytocin- or histamine-stimulated $[Ca^{2+}]_i$ signalling, cells were transfected with either negative control (100 nM), anti-arrestin2 (100 nM), anti-arrestin3 (10 nM) or both anti-arrestin2 and anti-arrestin3 siRNAs. Agonist-stimulated changes in $[Ca^{2+}]_i$ were monitored 48 h after transfection after addition of a single maximal oxytocin (100 nM) or histamine (100 μM) concentration. For both oxytocin and histamine, transfection with negative control siRNA had no effects on basal fluorescence ($[Ca^{2+}]_i$) values (Figure 2), or the magnitude or peak-plateau profiles of oxytocin- or histamine-stimulated $[Ca^{2+}]_i$ signals (data not shown). However, following siRNA depletion of either arrestin2 or arrestin3, peak-plateau oxytocin-stimulated $[Ca^{2+}]_i$ changes were significantly enhanced when compared with negative control transfected cells ($P < 0.01$ two-way ANOVA; Bonferroni's *post hoc* test). Combined depletion of both arrestin isoforms by co-transfection of anti-arrestin2 and anti-arrestin3 siRNAs failed to enhance oxytocin-induced $[Ca^{2+}]_i$ changes to any greater extent than by depletion of individual arrestin isoforms. While arrestin depletion (either individually or in combination) did not affect histamine-stimulated peak $[Ca^{2+}]_i$ changes (Figure 2E), the plateau phase was significantly enhanced siRNA-mediated arrestin3 suppression ($P < 0.01$ two-way ANOVA; Bonferroni's *post hoc* test). Knockdown of arrestin2 did not significantly affect the plateau phase of the histamine-stimulated Ca^{2+} response and failed to increase the effect seen on arrestin3 depletion (Figure 2E). These data suggest that arrestin2 and 3 regulate oxytocin receptor signalling, whilst arrestin3 alone is responsible for the regulation of H_1 receptor signalling.

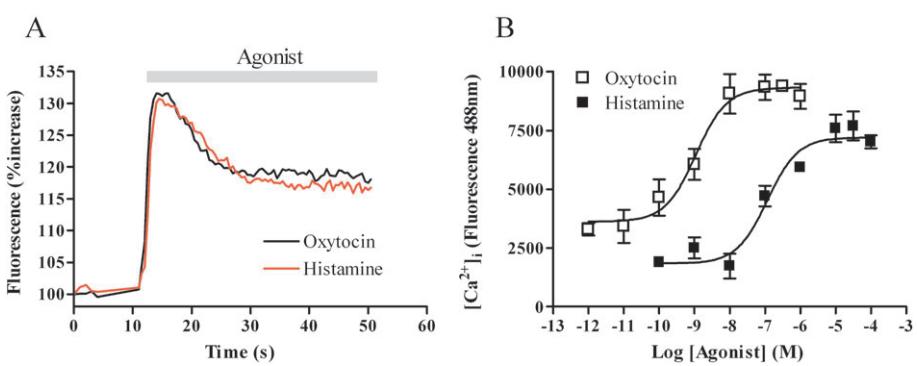

Figure 1

Characterization of histamine and oxytocin induced intracellular calcium concentration ($[Ca^{2+}]_i$) changes in myometrial cells. ULTR cells were loaded with Fluo4AM (3 μM, for 1 h) and agonist-induced $[Ca^{2+}]_i$ changes monitored using a NovoStar imaging system (see *Methods*). (A) Representative traces showing typical temporal profiles of $[Ca^{2+}]_i$ changes after stimulation with maximal concentrations of oxytocin (100 nM) or histamine (100 μM). (B) Concentration–response curves generated after stimulation with varying concentrations of oxytocin or histamine. Data are shown as means ± SEM from six separate experiments.

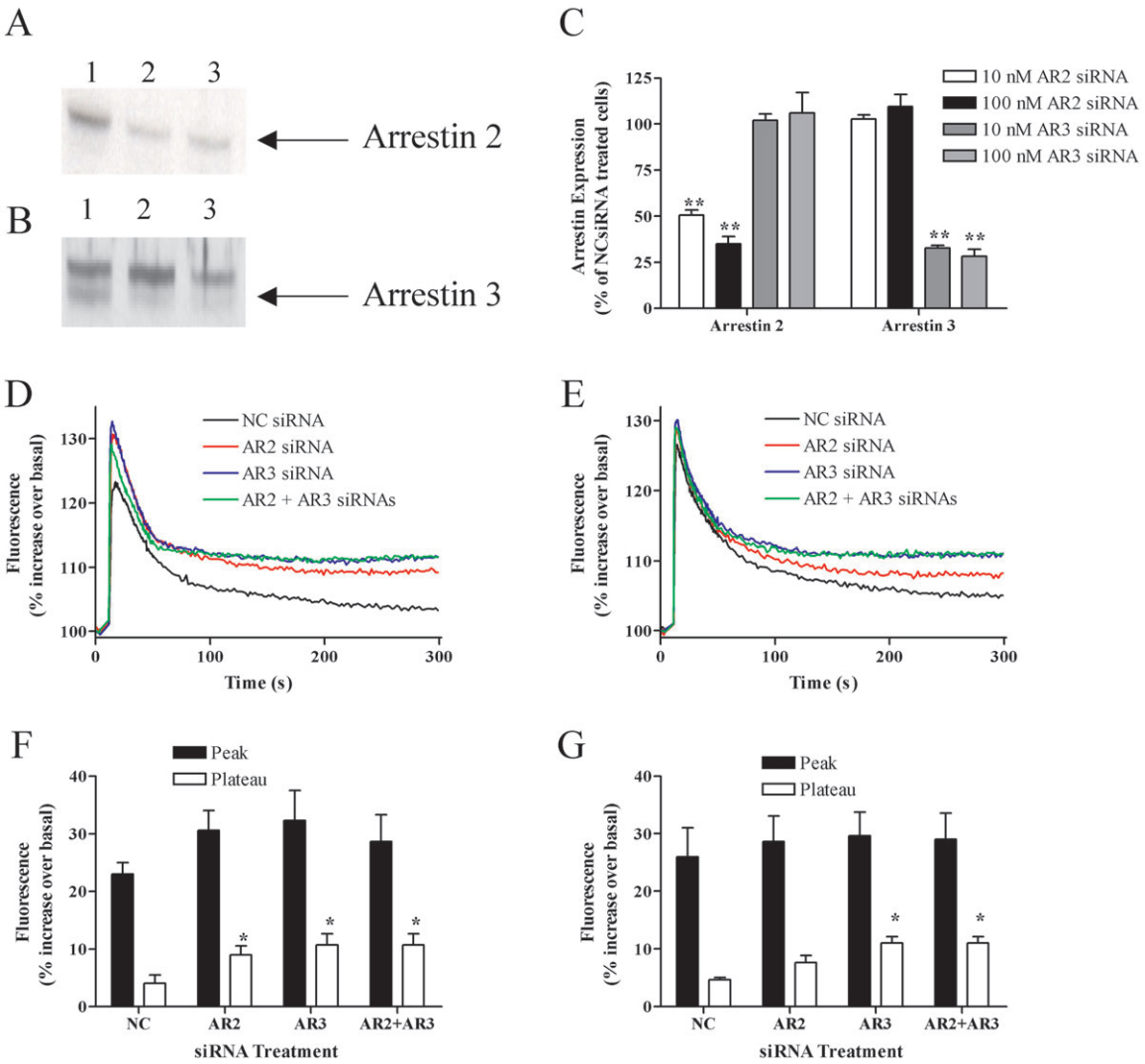

Figure 2

Arrestin depletion prolongs oxytocin- and histamine-induced Ca^{2+} signalling. ULTR cells were transfected with siRNAs against arrestin2 (AR2, 100 nM), arrestin3 (AR3, 10 nM) or negative control (NC, 100 nM) siRNA. After 48 h cells were lysed and arrestin expression levels determined via Western blotting (see *Methods*). (A) Representative immunblot showing arrestin2 expression following treatment with negative control (lane 1), anti-arrestin2 (lane 2) or both anti-arrestin2 and anti-arrestin3 (lane 3) siRNAs respectively. (B) Representative immunblot showing arrestin3 expression (the lower band) following transfection with negative control (lane 1), anti-arrestin3 (lane 2) or anti-arrestin2 and anti-arrestin3 (lane 3) siRNAs respectively. Cumulative data are also shown quantifying the extent of siRNA-mediated arrestin suppression in ULTR cells (C). Data are expressed as means ± SEM from four separate experiments. Significant differences seen following knockdown relative to negative control transfected cells are indicated as $^{**}P < 0.01$. Oxytocin (100 nM) (D) and histamine (100 μM)-stimulated (E) intracellular calcium concentration ($[Ca^{2+}]_i$) signals were examined in the presence or absence of arrestin2 or arrestin3 or both arrestins. Mean trace data are shown from $n = 3$ experiments and SEM are omitted for clarity. Cumulative data show peak and plateau $[Ca^{2+}]_i$ changes (at 200 s) following oxytocin (F) and histamine (G) stimulation taken from the temporal traces shown in (D) and (E). Oxytocin-stimulated plateau $[Ca^{2+}]_i$ levels were significantly enhanced ($^{*}P < 0.05$, one-way ANOVA, Bonferroni's *post hoc* test) following knockdown of either arrestin isoform, while histamine-stimulated plateau $[Ca^{2+}]_i$ levels were significantly enhanced ($^{*}P < 0.05$, one-way ANOVA, Bonferroni's *post hoc* test) only in the absence of arrestin3. Mean basal fluorescent $[Ca^{2+}]_i$-values for non-transfected cells (25564 ± 357) were unaltered following transfection with negative control (24685 ± 1151), anti-arrestin2 (24931 ± 399), anti-arrestin3 24338 ± 764) or both anti-arrestin3 and 3 (25185 ± 862) siRNAs (data are mean arbitrary fluorescent units ± SEM, for $n = 3$ experiments for each condition).

Effects of arrestin isoform depletion on oxytocin and H_1 receptor desensitization

We have previously determined that GRK2 (Willets *et al.*, 2008) and GRK6 (Willets *et al.*, 2009) are the respective key regulators of H_1 and oxytocin receptor desensitization in ULTR and primary human myometrial cell. These findings prompt the question of whether GRK-mediated phosphorylation leads to arrestin binding and what the consequences of this for downstream signalling are? To answer this question,

myometrial cells were co-transfected with eGFP-PH and either negative control (100 nM), anti-arrestin2 (100 nM) or anti-arrestin3 (10 nM) siRNAs. As expected application of our previously validated desensitization protocols highlighted markedly reduced R2 values compared with R1 for both histamine and oxytocin challenge. Reduction in the R2/R1 ratio is indicative of receptor desensitization and was comparable to that previously observed in the presence or absence of negative control siRNA (i.e. 50% for H_1 and 75% for oxytocin receptor responses (Willets *et al.*, 2008; 2009) (Figure 3A, D and G). Suppression of arrestin3, but not arrestin2 expression markedly reversed H_1 receptor desensitization (Figure 3B, C and G). In contrast, depletion of either arrestin isoform was equally efficient at reversing oxytocin receptor desensitization (Figure 3E–G). These findings highlight a key role for arrestin3 in the regulation of H_1 receptor desensitization whilst oxytocin receptor desensitization is mediated equally by both arrestin2 and arrestin3.

Characteristics of oxytocin- and histamine-mediated ERK1/2 phosphorylation

The effects of oxytocin and H_1 receptor activation on the ERK1/2 signalling pathway in ULTR cells were investigated by immunuoblotting techniques using antibodies for total and phospho-forms (p) of ERK1/2. Oxytocin induced a 200% increase over basal in pERK1/2, which peaked at 3 min and rapidly returned towards basal after 5 min. In contrast, histamine-stimulated pERK1/2 responses, whilst peaking at 3 min, were more prolonged, declining over the succeeding 15 min. Concentration–response analysis for pERK1/2 immunoreactivity at 3 min after agonist addition generated EC_{50} values of 1.3 nM for oxytocin [pEC_{50} (M) = 8.84 ± 0.18] and 7.1 μM for histamine [pEC_{50} (M) = 5.14 ± 0.21] (Figure 4C, D and F). In agreement with previous findings in ULTR cells (Willets *et al.*, 2008), histamine-stimulated pERK1/2 and phospho-p38 signals were blocked by the H_1 antagonist diphenhydramine (10 μM), but not by the H_2 receptor antagonist, cimetidine (10 μM, data not shown).

Differential effects of arrestin isoform depletion on oxytocin- and histamine-stimulated ERK1/2 signalling

For at least some GPCRs, arrestin proteins have well-documented roles as agonist-regulated adaptor scaffolds for cell surface receptor-to-ERK1/2 signalling (Ahn *et al.*, 2004b; DeWire *et al.*, 2007), although their potential role in myometrium has not yet been investigated. Here, we examined the effects of specific siRNA-targeted arrestin suppression on oxytocin- and histamine-stimulated ERK1/2 phosphorylation (Figure 4). Transfection of ULTR cells with negative control siRNA had no effect on basal pERK1/2 levels, or the magnitude or time course of oxytocin- or histamine-stimulated ERK1/2 responses compared with non-transfected cells (Figures 4 and 5). However, following siRNA-mediated depletion of arrestin2, a marked increase in oxytocin-mediated ERK1/2 activation was observed, with a doubling of the peak response, which was maintained over a more sustained time course (Figure 5A and C). Conversely, depletion of arrestin3 attenuated peak pERK1/2 levels, and virtually abolished any sustained signal (Figure 5A and C). As observed with oxytocin challenge, depletion of arrestin2 also enhanced both the peak and sustained phases of histamine-stimulated ERK1/2 activation (Figure 5B and D), although this effect was not as great as for oxytocin-stimulated responses. Strikingly, the histamine-evoked pERK1/2 response was completely absent after depletion of arrestin3 (Figure 5B and D). Previous reports highlight a role for PKC in mediating $G\alpha_{q/11}$ coupled receptor-ERK1/2 activation (Kim *et al.*, 2005; DeWire *et al.*, 2007). To assess whether PKC was involved in uterotonic-ERK1/2 responses in ULTR cells, either oxytocin (100 nM) or histamine (100 μM) was applied for 3 min, in the presence or absence of the PKC inhibitor Go6976 (1 μM, 15 min pre-incubation). PKC inhibition had no effect upon histamine-stimulated ERK signals; however, oxytocin-induced ERK1/2 phosphorylation was inhibited by a third (Figure 6A, B and E). Interestingly, both oxytocin- and histamine-evoked pERK1/2 responses appear heavily reliant on extracellular Ca^{2+}, because agonist-stimulated pERK1/2 signals were virtually undetectable when extracellular Ca^{2+} was removed from the assay buffer (Figure 6C–E). These data indicate that histamine-induced ERK1/2 phosphorylation is entirely reliant on the presence of arrestin3, and is enhanced following depletion of arrestin2. In contrast, the peak phase of oxytocin-induced ERK1/2 phosphorylation requires both PKC activity and the presence of arrestin3, whilst again oxytocin-induced ERK1/2 activation was enhanced in the absence of arrestin2.

Oxytocin- and histamine-stimulated phosphorylation of p38 MAPK

Oxytocin and histamine induced time- and concentration-dependent activation of p38 MAPK signalling (Figure 7). Oxytocin induced a maximal increase in phospho-p38 immunoreactivity after 5 min, which gradually declined to basal levels within 25 min (Figure 7A and E), whilst histamine induced maximal p38 phosphorylation after 10 min, declining to basal by 25 min (Figure 7B and E). Concentration–response analysis at the respective peak time points for the two agonists revealed EC_{50} values of 0.23 nM [pEC_{50} (M) = 9.65 ± 0.2] for oxytocin (Figure 7C and F) and 89 nM [pEC50 (M) = 7.05 ± 0.3] for histamine (Figure 7D and F) respectively.

Differential effects of arrestin isoform depletion on oxytocin- and histamine-stimulated p38 MAPK signalling

Although less widely investigated, there is accruing evidence of an intermediary role for arrestins linking GPCRs to p38 MAPK (Sun *et al.*, 2002; Bruchas *et al.*, 2006; Gong *et al.*, 2008). To investigate the role that arrestins might play in the oxytocin- and/or histamine-mediated p38 MAPK signalling, we examined the time course profiles of p38 phosphorylation stimulated by both receptors following siRNA-mediated depletion of arrestin2 or 3. When compared with negative control siRNA-transfected cells, the peak and sustained phases of oxytocin-induced p38 signalling were significantly enhanced in ULTR cells transfected with anti-arrestin2 or anti-arrestin3 siRNAs (Figure 8A and C). In the presence of negative control or arrestin2 siRNAs, the profile of histamine-induced p38 activation was similar to that observed in non-transfected cells, whereas arrestin3 depletion markedly

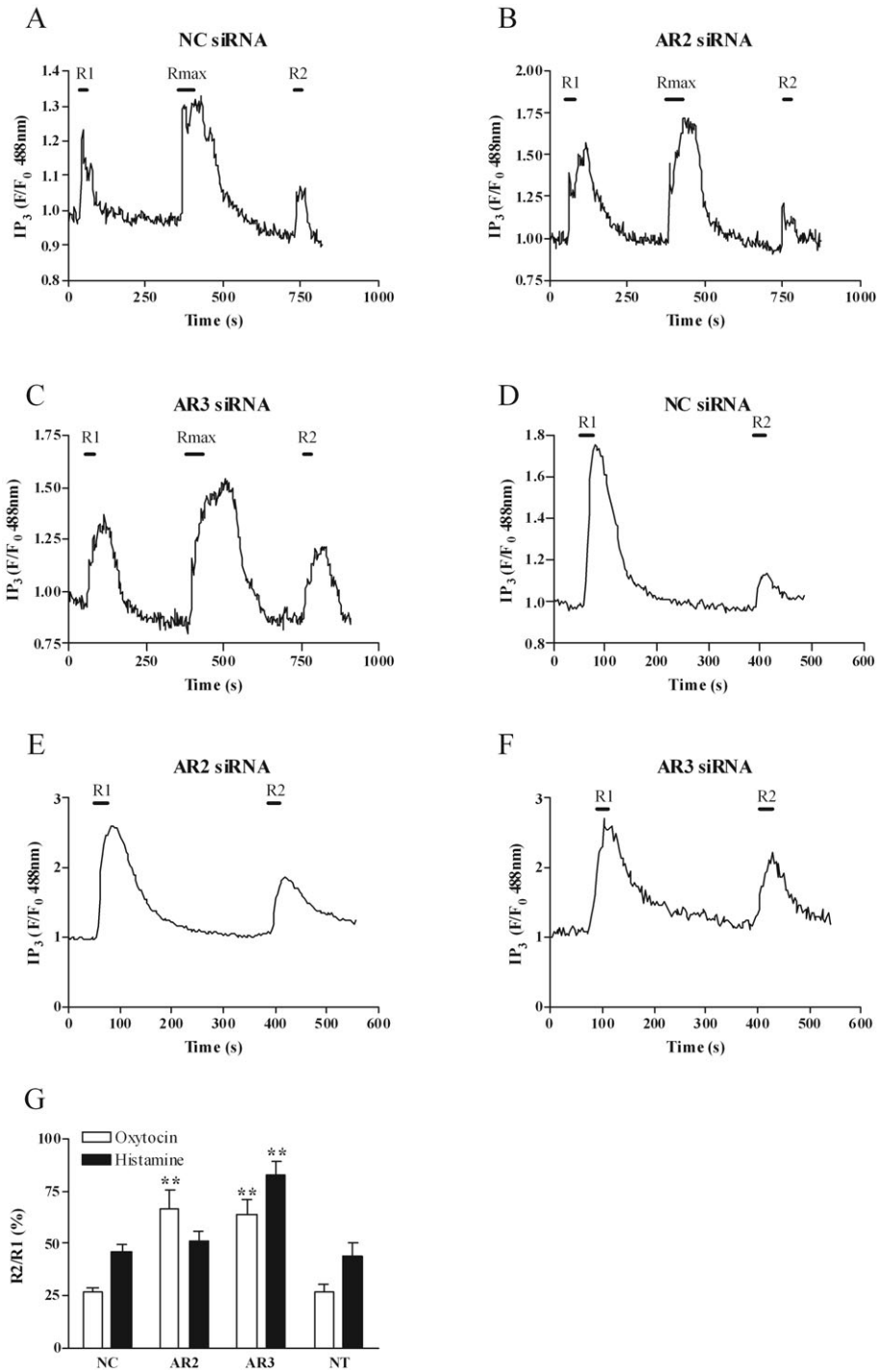

Figure 3

Suppression of arrestin expression prevents oxytocin and H_1 receptor desensitization. Cells were co-transfected with e-GFP-tagged pleckstrin homology domain of phospholipase Cδ (0.5 μg) and either anti-arrestin2 (AR2, 100 nM), anti-arrestin3 (AR3, 10 nM) or negative control (NC, 100 nM) as described in the *Methods* section. After 48 h, H_1 receptor desensitization was assessed using the standard desensitization protocol (R1, R2 = 10 μM histamine for 30 s; R_{max} = 100 μM histamine for 60 s) and oxytocin receptor desensitization assessed using an alternative desensitization protocol whereby 100 nM oxytocin was applied for 30 s during R1 and R2 with 5 min washout period. Representative traces show the effects of negative control (A) anti-arrestin2 (B) and anti-arrestin3 (C) siRNA treatment on H_1 receptor during the R1/R_{max}/R2 protocol. Representative traces also display the effects of negative control (D) anti-arrestin2 (E) and anti-arrestin3 (F) siRNA treatment on oxytocin receptor responses during the alternative R1/R2 protocol. Cumulative data (G) show a significant (**$P < 0.01$, one-way ANOVA, Dunnett's *post hoc* test) decrease in the extent of H_1 receptor desensitization after depletion of endogenous arrestin3 and oxytocin receptor desensitization after suppression of arrestin2 or arrestin3 expression. Data are presented as means ± SEM for the % change in R2/R1 ratio for between 7 and 17 cells from at least six separate experiments. IP_3, inositol 1,4,5-trisphosphate.

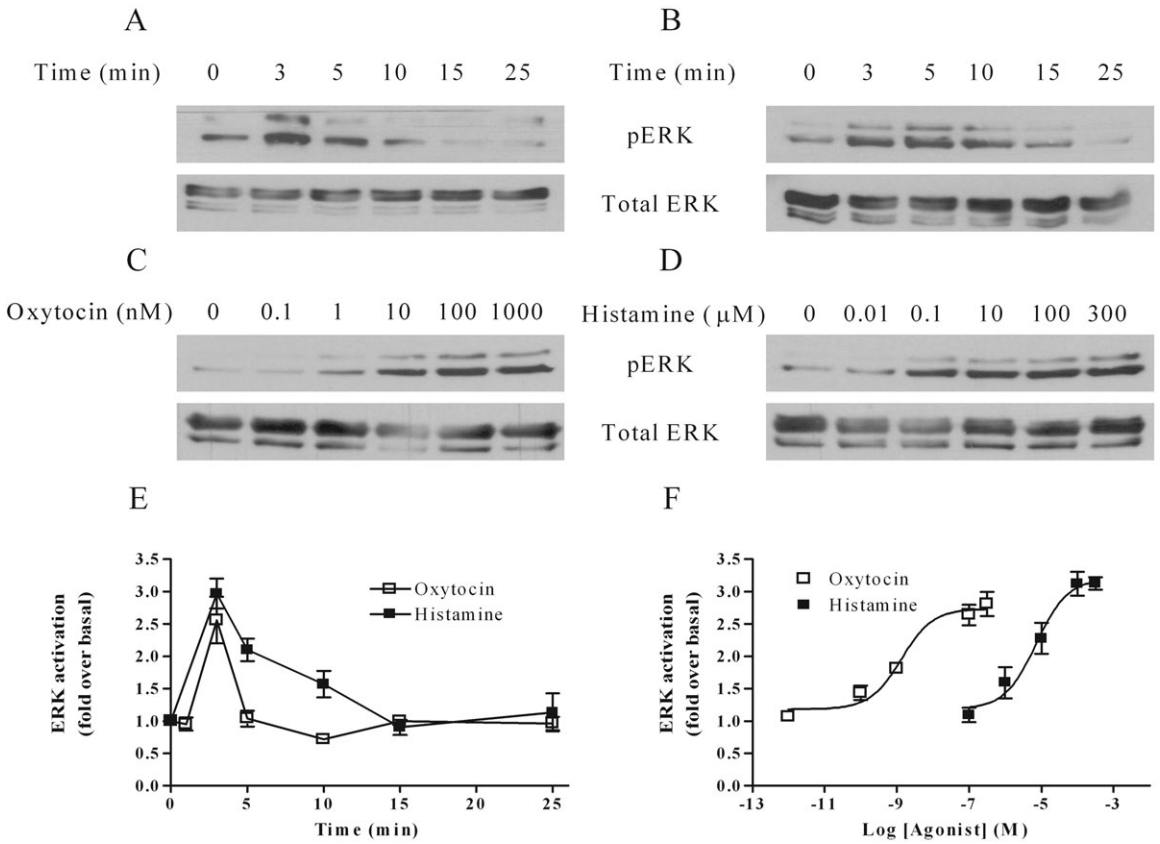

Figure 4

Time courses and concentration-dependencies for oxytocin- and histamine-stimulated extracellular signal-regulated kinase (ERK)1/2 phosphorylation. ULTR cells were deprived of serum for 24 h prior to agonist stimulation, and threonine- and tyrosine-phosphorylated extracellular signal-regulated kinase (pERK)1/2 levels determined by standard immunoblotting techniques (A–D, upper panels). To ensure that all samples contained the same levels of protein, total ERK levels were determined by running additional gels in parallel with the detection of pERK using an anti-ERK1 antibody (A–D, lower panels). Representative immunoblots show time courses for responses to oxytocin (100 nM) (A) or histamine (100 μM) (B), and concentration-dependencies for oxytocin (C) and histamine (D). Concentration-dependencies were determined at the peak ERK1/2 phosphorylation time point (3 min). Mean data (± SEM) for time courses (E) and concentration-dependencies (F) are shown for $n = 3$ experiments.

increased both the peak and sustained phases of histamine-stimulated p38 signalling (Figure 8B and D). Furthermore, mean basal p38 phosphorylation levels were similar in non-transfected and siRNA-transfected cells (Figure 8). Our findings suggest that arrestin proteins play a key role in negatively regulating uterotonic p38 MAPK signalling in myometrial cells. Moreover, both arrestin isoforms are equally adept at limiting oxytocin-induced p38 signals, whereas arrestin3 appears to be selectively involved in modulating histamine-evoked signals.

Discussion and conclusions

Here we investigated how the uterotonins, oxytocin and histamine cause their respective GPCRs (oxytocin and H_1) to recruit arrestin isoforms and how this contributes to the desensitization of G protein-mediated signalling, whilst also contributing to the recruitment of alternate receptor-driven signalling mechanisms within myometrial smooth muscle cells. Oxytocin-mediated myometrial contraction has an established role in the progression of labour (Blanks and Thornton, 2003; Zingg and Laporte, 2003). Histamine, most likely released from infiltrating or resident uterine mast cells (Massey *et al.*, 1991; Bytautiene *et al.*, 2004b), can also induce myometrial contractions via H_1 receptor activation (Rudolph *et al.*, 1993; Bytautiene *et al.*, 2003), a process implicated in preterm labour (Bytautiene *et al.*, 2003; 2004a,b). Regulation of GPCR signalling is complex, involving many different regulatory processes; however, GPCR desensitization, mediated by GRK and arrestin proteins, is fundamental in preventing prolonged or inappropriate signalling (Willets *et al.*, 2003; Premont and Gainetdinov, 2007). We previously identified GRK6 and GRK2 as the respective primary initiators of myometrial oxytocin and H_1 receptor desensitization (Willets *et al.*, 2008; 2009), findings that suggest these receptors are also likely to recruit arrestin proteins. Although H_1 receptor–arrestin interactions are presently underexplored, a number of studies have demonstrated oxytocin receptor–arrestin interactions (Oakley *et al.*, 2001; Hasbi *et al.*, 2004; Smith

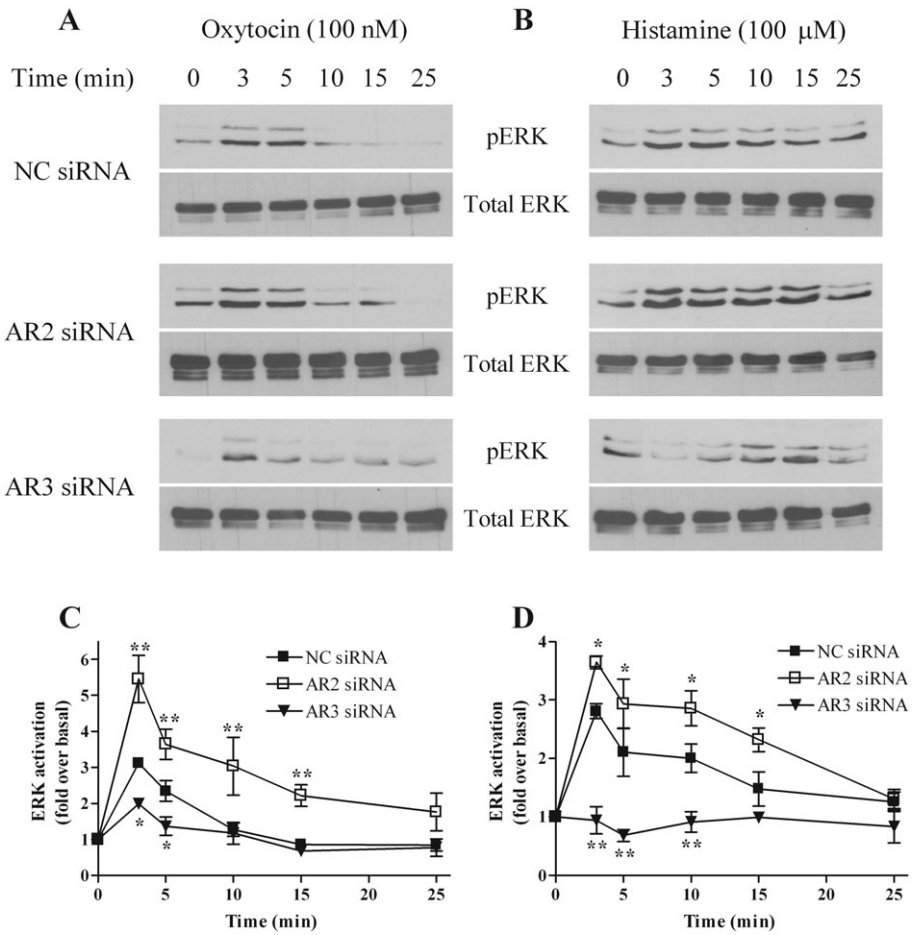

Figure 5

Arrestins differentially regulate oxytocin- and histamine-stimulated extracellular signal-regulated kinase (ERK)1/2 signalling. ULTR cells were transfected with negative control (NC, 100 nM), anti-arrestin2 (AR2, 100 nM) or anti-arrestin3 (AR3, 10 nM) siRNA for 48 h. Cells were serum-starved for the last 24 h prior to agonist stimulation for the times stated. ULTR cells were lysed and threonine- and tyrosine-phosphorylated extracellular signal-regulated kinase (pERK)1/2 levels determined by standard immunoblotting techniques (upper panels). To ensure that all samples contained the same levels of protein, total ERK levels were determined by running additional gels in parallel with the detection of pERK using an anti-ERK1 antibody (A–D, lower panels). Representative immunoblots show the effects of arrestin depletion on oxytocin (A) or histamine (B)-stimulated pERK1/2 responses. Cumulative densitometric analysis of oxytocin (C) or histamine (D)-stimulated ERK1/2 phosphorylation. Data are shown means ± SEM of $n = 4$ experiments. Depletion of arrestin3 significantly attenuated both oxytocin and histamine-stimulated pERK1/2 responses, whilst arrestin2 depletion significantly enhanced oxytocin and histamine-stimulated ERK1/2 signals (*$P < 0.05$, **$P < 0.01$, two-way ANOVA, Bonferroni's *post hoc* test) when compared with negative control-treated cells. Mean basal pERK1/2 levels were similar in non-transfected (11722 ± 1592), to negative control (12937 ± 2970), anti-arrestin2 (11837 ± 1961) and anti-arrestin3 (12743 ± 2792) transfected cells (data are mean absorbance units·mm^{-2} ± SEM, $n = 4$).

et al., 2006; Conti *et al.*, 2009). However, all previous studies to date have been performed in model cell (HEK293/COS-7) systems with recombinantly expressed oxytocin receptors. Therefore, our study is the first to assess the roles of arrestin proteins in oxytocin and H_1 receptor regulation within the myometrium.

Initial studies assessed the effects of siRNA-mediated selective depletion of arrestin2 and 3 isoforms on oxytocin and H_1 receptor signalling and desensitization. By examining a simple signalling readout ($[Ca^{2+}]_i$) and by using previously validated protocols to assess how prior agonist exposure affects subsequent responses to rechallenge with agonist [assessed at a single cell level using the eGFP-PH biosensor of phosphoinositide turnover; (Nelson *et al.*, 2008; Willets *et al.*, 2008)] we have been able to demonstrate different arrestin isoform dependencies of the two receptors. Thus, while knockdown of either arrestin2 or 3 augmented oxytocin-mediated Ca^{2+} responses and attenuated the extent of agonist-mediated oxytocin receptor desensitization, only arrestin3 knockdown had similar effects on H_1 receptor signalling and desensitization. Previous studies also demonstrate an agonist- and GRK-dependent interaction of recombinant oxytocin receptors and arrestin3 in HEK293/COS-7 cell backgrounds (Oakley *et al.*, 2001; Hasbi *et al.*, 2004; Smith *et al.*, 2006), with one study pinpointing two *C*-terminal Ser-Ser-Ser (368–370/377–379) clusters as the oxytocin receptor domains undergoing GRK phosphorylation and arrestin3 binding (Oakley *et al.*, 2001). However, these

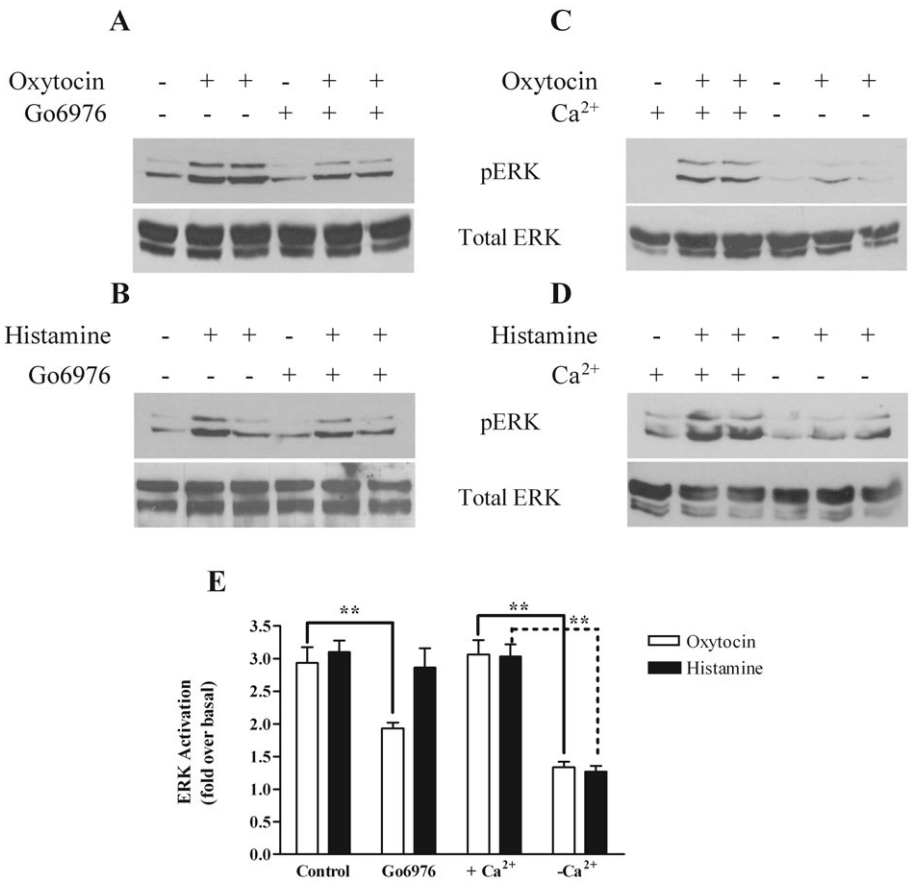

Figure 6

Ca^{2+} and protein kinase C (PKC) dependence of oxytocin- and histamine-stimulated threonine- and tyrosine-phosphorylated extracellular signal-regulated kinase (pERK)1/2 responses. Representative immunoblots are displayed showing (A) oxytocin (100 nM) and (B) histamine (100 μM)-stimulated pERK1/2 signals in the presence or absence of the PKC inhibitor Go6976 (1 μM, for 15 min prior to agonist challenge). Representative immunoblots (upper panels) are shown for (C) oxytocin and (D) histamine-stimulated pERK1/2 responses generated in the presence or nominal absence of extracellular Ca^{2+} (i.e. in nominally Ca^{2+}-free Krebs). To ensure that all samples contained the same levels of protein, total extracellular signal-regulated kinase (ERK) levels were determined by running additional gels in parallel with the detection of pERK using an anti-ERK1 antibody (A–D, lower panels). Cumulative densitometric analysis pERK1/2 responses (E) show that removal of extracellular Ca^{2+} dramatically inhibits oxytocin- and histamine-evoked pERK1/2 immunonoreactivity (**$P < 0.01$, one-way ANOVA, Dunnett's *post hoc* test). PKC inhibition partially inhibited oxytocin- (**$P < 0.01$, one-way ANOVA, Dunnett's *post hoc* test), but not histamine-induced pERK1/2 responses. Data are shown as means ± SEM of $n = 4$ experiments.

studies all focused on oxytocin receptor–arrestin3 interactions and did not investigate the possibility of an oxytocin receptor–arrestin2 interaction. Here, we provide novel data on oxytocin receptor regulation by both arrestin2 and 3 in myometrial cells endogenously expressing this GPCR subtype, as well as the first evidence that H_1 receptor signalling/desensitization is modulated by arrestin3, but not arrestin2.

An emerging theme over the past 10 years has been a realization that whilst arrestin binding results in GPCR uncoupling from G proteins and desensitization/internalization, it can also allow the receptor-arrestin complex to fulfil alternate signalling functions within the cell (Luttrell and Lefkowitz, 2002; DeWire *et al.*, 2007; Gesty-Palmer and Luttrell, 2008). To investigate (some of) the signalling consequences of oxytocin and H_1 receptor-arrestin binding, we assessed how the selective depletion of arrestin2 or 3 affects the time courses of agonist-stimulated ERK and p38 MAPK phosphorylation in human myometrial ULTR cells. Initial experiments established that oxytocin and histamine stimulated both ERK and p38 MAPK responses with similar peak increases, but somewhat different time courses of response. Similar findings have previously been obtained for oxytocin in myometrial preparations (Robinson and Dickenson, 2001; Zhong *et al.*, 2003; Devost *et al.*, 2008) and H_1 receptor-mediated ERK1/2 and p38 MAPK activation has been reported in non-myometrial preparations (Robinson and Dickenson, 2001). Our data confirm a role for PKC in oxytocin-induced activation of ERK1/2 signals (Zhong *et al.*, 2003); however, PKC appears to play a lesser role in mediating oxytocin ERK1/2 signalling in non-pregnant ULTR, than immortalized pregnant PHM1 myometrial cells (Zhong *et al.*, 2003). These differences in oxytocin ERK1/2 signalling may reflect physiological changes in oxytocin receptor regulation induced by pregnancy. Indeed, removal of extracellular

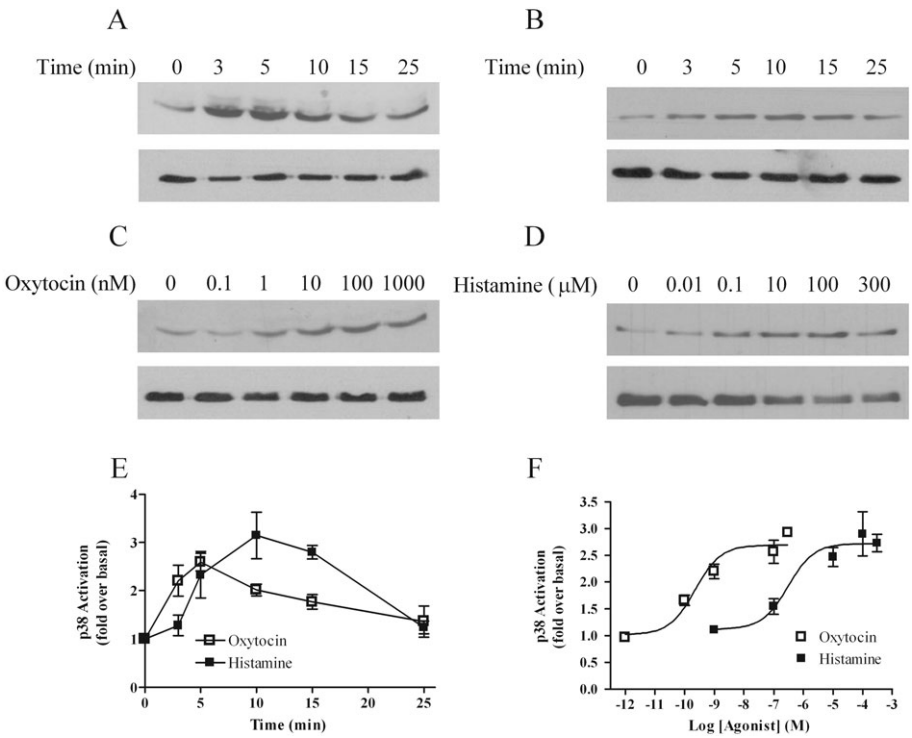

Figure 7

Time course and concentration-dependence of oxytocin- and histamine-stimulated p38 mitogen-activated protein kinase phosphorylation. Cells were deprived of serum for 24 h prior to agonist stimulation, and phospho-p38 (pp38) levels determined by standard immunoblotting techniques (A–D upper panels). To ensure that all samples contained the same levels of protein, total p38 levels were determined by running additional gels in parallel with the detection of p38 using an anti-p38 antibody (A–D, lower panels). Representative immunoblots show the time courses of oxytocin (100 nM) (A), and histamine (100 μM) (B) and concentration-dependence of oxytocin- (C) and histamine- (D) stimulated p38 activation. Concentration-dependencies were determined at the peak p38 phosphorlyation time point (oxytocin 5 min; histamine 10 min). Mean data (± SEM) for time courses (E) and concentration-dependencies (F) are shown for n = 3–5 separate experiments.

Ca^{2+} induced a far more pronounced inhibition of oxytocin-stimulated ERK1/2 signalling in non-pregnant myometrial (ULTR) cells. Moreover, histamine-stimulated ERK1/2 signals were abolished in the absence of extracellular Ca^{2+}, and were PKC-independent. Collectively, these data infer that Ca^{2+} entry is the main driving force behind the peak phase of oxytocin and histamine ERK1/2 signalling in ULTR cells, which mirrors previous findings mediated by the gonadotropin-releasing hormone receptor in LβT-2 cells (Bonfil *et al.*, 2004).

Existing work on GPCR-bound arrestins acting as signalling scaffolds has often focused on how this mechanism complements others utilized by GPCRs to activate MAPK signalling (e.g. those involving small GTPases, transactivation, growth-factor-shedding, etc.). For example, arrestins have been shown to scaffold ERK1/2 to the GPCR, leading to retention of active ERK1/2 within the cytoplasm and prolongation of signalling (DeWire *et al.*, 2007). Here we demonstrate that selective arrestin3 knockdown in ULTR cells markedly attenuates ERK1/2 activation by either oxytocin or histamine. Previous studies have often reported a different time course of arrestin-dependency, with initial peak responses being generally less dependent on GPCR-arrestin-ERK1/2 signalling than the sustained phase (Ahn *et al.*, 2004a; DeWire *et al.*, 2007). Our oxytocin data appear to concur with this scenario because both PKC and arrestin3 appear to contribute equally to peak ERK1/2 activation, while sustained ERK phosphorylation is arrestin3-dependent. By contrast, we show that arrestin3 depletion completely ablates histamine-mediated ERK1/2 signalling and markedly attenuates the oxytocin-stimulated response at the earliest time point investigated. For both oxytocin- and H_1 receptor-stimulated ERK1/2 responses arrestin2 knockdown resulted in enhanced initial responses that were evident across the first 15 min of the time course studied here. These data echo those reported in the earliest arrestin/siRNA studies, where knockdown of arrestin3 decreased, and arrestin2 enhanced, AT_{1A} and V_2 vasopressin receptor-ERK1/2 signalling in a HEK293 cell background (Ahn *et al.*, 2004b; Ren *et al.*, 2005). The enhancement of oxytocin receptor-ERK1/2 signalling seen when arrestin2 is selectively depleted correlates with the observed decrease in receptor desensitization, and desensitization/internalization of this receptor subtype certainly occurs very rapidly in myometrial derived cells (Conti *et al.*, 2009). However, a similar explanation cannot be elaborated for the qualitatively similar enhancement of H_1 receptor-ERK1/2 signalling as desensitization of this GPCR was not significantly affected by arrestin2 knockdown. Therefore, the precise mechanism(s) whereby arrestin2 knockdown facilitates both oxytocin and H_1 receptor-ERK1/2 signalling have yet to be established.

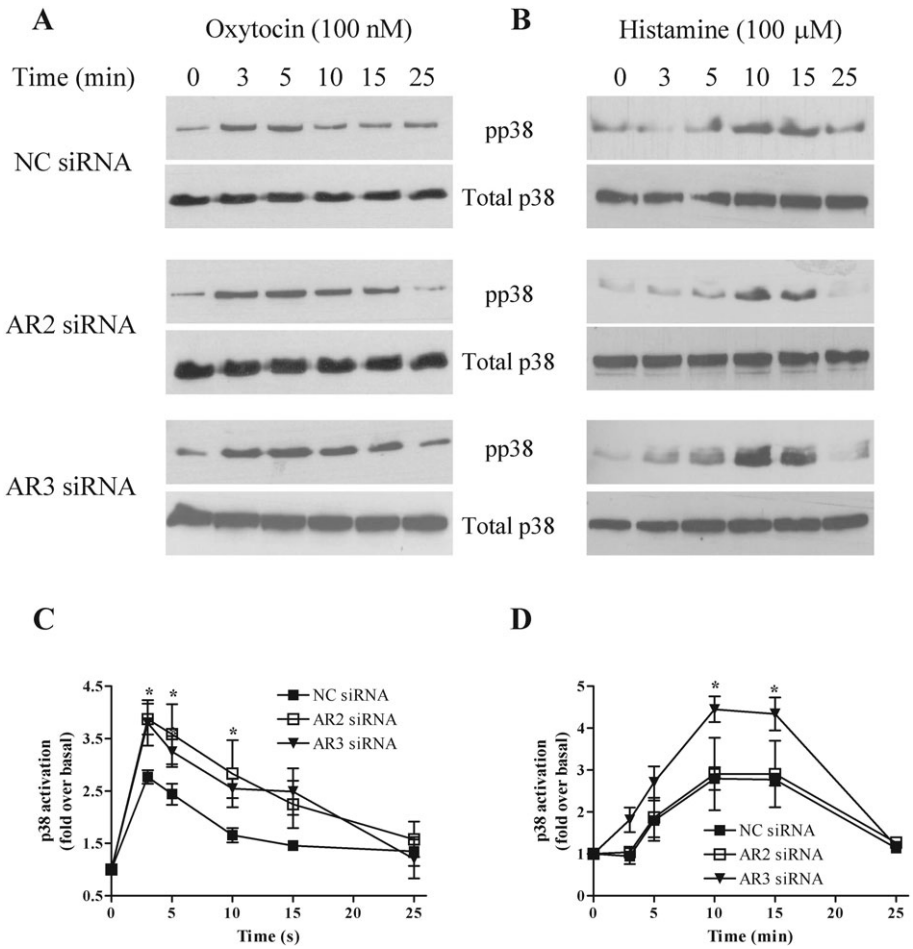

Figure 8

Arrestins differentially regulate oxytocin and histamine-stimulated extracellular signal-regulated kinase (ERK) signalling. ULTR cells were transfected with negative control (NC 100 nM), anti-arrestin2 (AR2, 100 nM) or anti-arrestin3 (AR3, 10 nM) siRNA for 48 h. Cells were deprived of serum for the last 24 h prior to agonist stimulation for the times stated. ULTR cells were lysed and phospho-p38 immunoreactivity determined by standard immunoblotting techniques (upper panels). To ensure that all samples contained the same levels of protein, total p38 levels were determined by running additional gels in parallel with the detection of phospho-p38 using an anti-p38 antibody (A–D, lower panels). Representative immunoblots show the effects of arrestin depletion on oxytocin (100 nM) (A) or histamine (100 μM) (B) stimulated p38 responses. Cumulative densitometric analysis of oxytocin (C) or histamine (D) stimulated p38 phosphorylation. Data are shown as means ± SEM of $n = 4$ experiments. Depletion of arrestin3 significantly enhanced both oxytocin- and histamine-stimulated p38 responses, whereas arrestin 2 depletion significantly enhanced oxytocin- but not histamine-stimulated p38 responses (*$P < 0.05$, **$P < 0.01$, two-way ANOVA, Bonferroni's *post hoc* test), when compared with negative control-treated cells. Mean basal p38 phosphorylation levels were similar in non-transfected (9358 ± 1203), to negative control (8245 ± 2233), anti-arrestin2 (8677 ± 2023) and anti-arrestin3 (9546 ± 2683) transfected cells (data are mean absorbance units·mm^{-2} ± SEM, $n = 4$).

Although less studied, GPCR coupling to another MAPK signalling pathway has also been reported to occur via arrestin-dependent mechanisms. As for the GPCR-ERK1/2 signalling pathway, it is likely that arrestins will be one of a number of different mechanisms that can link activated GPCRs to p38 MAPK signalling. However, preliminary experiments demonstrated that both oxytocin and H_1 receptor subtypes can increase p38 MAPK phosphorylation in ULTR cells, albeit with somewhat different time courses of activation. Selective arrestin2 or 3 depletion had similar effects on the oxytocin receptor-p38 MAPK signalling, enhancing p38 MAPK phosphorylation, suggesting that each isoform normally suppresses rather than facilitates oxytocin receptor coupling to p38 MAPK. For H_1 receptor-p38 MAPK signalling arrestin3 knockdown enhanced the response, whilst arrestin2 knockdown was without effect. Interestingly, none of the manipulations of arrestin expression altered the overall time courses of p38 phosphorylation stimulated by oxytocin or histamine (see Figure 8C and D).

Previous studies that have manipulated cellular arrestin levels have generally reached different conclusions with respect to the roles of arrestins in p38 MAPK activation. Indeed, Sun *et al.* (2002) showed that arrestin-3 overexpression enhanced, whilst antisense or siRNA knockdown of arrestin3 attenuated, both CXCR4 and CCR5 chemokine receptor signalling to p38 MAPK in HEK293 cells. Within a

native cell background, Bruchas *et al.* (2006) showed that arrestin3 can act as a scaffold for p38 MAPK signalling and demonstrated that siRNA-knockdown of arrestin3 attenuated κ-opioid receptor signalling to this readout. These studies focused on manipulating cellular arrestin3 levels and no conclusions can be drawn with respect to isoform-selective arrestin effects within these pathways. More recently, a β_2-adrenoceptor–arrestin2 (but not arrestin3) interaction was proposed to act as a proximal scaffold eventually leading (via Rac1/NADPH oxidase-dependent intermediate steps) to p38 MAPK activation (Gong *et al.*, 2008). Collectively, these studies highlight a positive intermediary role for arrestin2 or 3 in GPCR coupling to p38 MAPK. Conversely, ablation of arrestin2 and 3 isoforms in double-knockout mouse embryonic fibroblasts dramatically enhanced CXCR2-stimulated p38 MAPK signalling (Zhao *et al.*, 2004), again with an intermediary role of Rac1/NADPH oxidase being implicated. Our data are mostly in agreement with the latter study and suggest that the failure of arrestin isoform depletions to suppress p38 MAPK signalling argues against arrestins acting as p38 scaffolds for oxytocin or histamine H_1 receptors in myometrium. Instead, enhanced p38 MAPK signalling following selective arrestin2 or 3 knockdown most likely reflects the decreased ability to desensitize oxytocin and histamine signalling in the absence of these proteins, suggesting that oxytocin and H_1 receptor coupling to p38 MAPK requires the receptor to remain at the cell surface.

In summary, this study builds on our previous work defining the GRK isoenzymic specificity of receptor desensitization by now identifying an important role for arrestin protein isoforms in the acute desensitization of the contractile myometrial oxytocin and H_1 receptors. Acute regulation of GPCR-mediated contractile signalling may add an additional 'brake' preventing inappropriate myometrial contraction, which in terms of H_1 receptor activation may be brought about through infection-induced mast cell degranulation (Massey *et al.*, 1991; Bytautiene *et al.*, 2004b). Furthermore, acute desensitization of oxytocin receptor signalling is likely to play an important role in the timing of myometrial contractions during labour, especially because uterine contractions cease despite the presence of circulating oxytocin. In addition, we have identified arrestin isoforms as key regulators of oxytocin- and histamine-stimulated MAPK signalling, which, considering the plethora of physiological processes mediated by MAPK signalling pathways, indicates that arrestins potentially play a significant role influencing myometrial sensitivity to external stimuli and fine tuning myometrial contractility. Indeed, MAPK signalling is reported to play a key role in the up-regulation of cyclooxygenase-2 expression (Bartlett *et al.*, 1999; Molnar *et al.*, 1999), highlighting a potential role for arrestins in the control of prostaglandin production and labour induction.

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Conflict of interest

None.

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