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Bradykinin-activated contractile signalling pathways in human myometrial cells are differentially regulated by arrestin proteins

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Bradykinin is associated with infections and inflammation, which given the strong correlation between uterine infection and preterm labour may imply that it could play a role in this process. Therefore, we investigated bradykinin signalling, and the roles that arrestin proteins play in their regulation in human myometrial cells. Bradykinin induced rapid, transient intracellular Ca^{2+} increases that were inhibited following B_2 receptor (B_2R) antagonism. Arrestin2 or arrestin3 depletion enhanced and prolonged bradykinin-stimulated Ca^{2+} responses, and attenuated B_2R desensitisation. Knockdown of either arrestin enhanced B_2R-stimulated ERK1/2 signals. Moreover, depletion of either arrestin elevated peak-phase p38-MAPK signalling, yet only arrestin3 depletion prolonged B_2R-induced p38-MAPK signals. Arrestin2-knockdown augmented bradykinin-induced cell movement. Bradykinin stimulates pro-contractile signalling mechanisms in human myometrial cells and arrestin proteins play key roles in their regulation. Our data suggest bradykinin not only acts as an utertonin, but may also have the potential to enhance the contractile environment of the uterus.

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1. Introduction

Bradykinin is a member of the kinin family, potent pro-inflammatory peptides which are stored as precursor peptides in the plasma membranes of cells (Blaes and Girolami, 2013). Kinins are liberated into the circulation following cleavage by serine proteases called kallikreins, a process that is greatly enhanced during bacterial infections (Blaes and Girolami, 2013). Interestingly, the presence of the kallikrein–kinin system in the human endometrium and myometrium (Clements et al., 1994) suggests that the uterus has the capacity to produce pro-inflammatory agents such as bradykinin in response to bacterial infections. Moreover, as there is a strong correlation between infection and preterm labour (Goldenberg et al., 2008), and that bradykinin is known to induce myometrial contraction (Janicek et al., 2007; Senior and Whalley, 1976), it is possible that bradykinin signalling may play a part in the induction of myometrial contraction not only secondary to infectious stimuli, but also conceivably in the normal parturition process. Indeed, in rats it has been suggested that the actions of bradykinin are mediated through the bradykinin B_2 receptor (B_2R) (Murone et al., 1999), which recruits $G\alpha_q$ and/or $G\alpha_i$ to activate phospholipase C (PLC) and inhibit adenylyl, increasing intracellular ($[Ca^{2+}]_i$) and reducing cyclic AMP concentrations, respectively (Marceau et al., 1998; Yusuf et al., 2000). Furthermore, B_2R receptor activation can induce MAPK signalling (Blaukat et al., 1999; Fleming et al., 1995), and activate phospholipase A_2, liberating arachidonic acid and subsequently increasing prostaglandin synthesis (Leeb-Lundberg et al., 2005). Activation of any or all of these signalling pathways may explain why bradykinin induces uterine contractions (Trzeciak et al., 2000; Wassdal et al., 1998).

The majority of bradykinin actions are mediated through two G protein-coupled receptors (GPCR) termed B_1 and B_2, which have little sequence homology (*e.g.* 36% for human bradykinin receptors). In general B_2 receptors (B_2R) are constitutively expressed in most tissues including smooth muscle (Couture et al., 2001), whereas, although B_1 expression is negligible in healthy cells its expression is inducible, being dramatically enhanced as a result of tissue injury, infection, inflammation or anoxia (Marceau et al., 1998).

Despite activating many of the same signalling pathways, the kinetics of B_1 and B_2 receptor signalling are very different. B_2R activation is typically transient, desensitising rapidly (Faussner et al., 1998), whilst B_1 receptor signalling is prolonged due to its relative insensitivity to phosphorylation and desensitisation (Blaukat et al., 1999). GPCR desensitisation is typically mediated through the action of G protein-coupled receptor kinases (GRK), which phosphorylate agonist-occupied receptors at serine and/or threonine moieties

Abbreviations: B_2R, bradykinin B_2 receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; eGFP-PH, e-GFP-tagged pleckstrin homology domain of phospholipase Cδ; pERK, threonine- and tyrosine-phosphorylated extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

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(Willets et al., 2003). GRK phosphorylation promotes the recruitment of arrestin proteins, whose binding physically blocks interaction of GPCR and G proteins, prior to promoting receptor internalisation (DeWire et al., 2007; Lamb et al., 2001; Willets et al., 2003). Indeed, data from model cell systems indicate that B_2R are substrates for several GRKs (Blaukat et al., 2001). As GRK mediated phosphorylation promotes arrestin interaction, it is also not surprising that arrestin proteins are reported to regulate exogenously expressed B_2R signalling in HEK293 cells (Zimmerman et al., 2011). Previous data from rats highlight the presence of B_2R on myometrial cells (Murone et al., 1999), activation of which would explain why bradykinin induces uterine contractions (Trzeciak et al., 2000; Wassdal et al., 1998). Notwithstanding these findings, presently little is known regarding the consequences of and regulatory mechanisms involved in bradykinin receptor signalling in the myometrium. Here we show that bradykinin activates pro-contractile signalling pathways within the human myometrium, and characterise the molecular mechanisms underlying bradykinin receptor regulation.

2. Materials and methods

2.1. Cell culture

The immortalised human ULTR myometrial cell line (Perez-Reyes et al., 1992) was cultured in Dulbecco's minimal essential medium, supplemented with 10% foetal calf serum, penicillin (100 IU ml^{-1}), streptomycin (100 µg ml^{-1}), and amphotericin B (2.5 µg ml^{-1}) and Glutamax-1. Cells were maintained under humidified conditions at 37 °C, in air/5% CO_2.

2.2. siRNA targeted arrestin and GRK depletion

Endogenous arrestin2 or arrestin3 were targeted for depletion using the following previously characterised (Brighton et al., 2011a, 2011b) siRNA constructs designed to target either human arrestin2 (ARRB1) (5'-GGAGAUCUAUUACCAUGGtt-3') or arrestin3 (ARRB2) siRNA (5'-CGAACAAGAUGACCAGGUAtt-3') (Ambion, Applied Biosystems, Warrington, UK). For MAPK and single cell imaging experiments ULTR cells (150,000 per well) were transfected with either negative control (non-targeting 100 nM), anti-arrestin2 (100 nM), or anti-arrestin3 (10 nM) siRNA constructs using the Interferin™ transfection reagent (Polyplus, NY, USA), as per manufacturers' instructions. Endogenous GRK proteins were targeted for depletion using the following previously characterised (Willets et al., 2009) siRNA constructs designed to target either GRK2 (5-GGCAGCCAGUGACCAAAAAtt-3), GRK3 (5-GGAACUUCUUUCCUGUUCAtt-3), GRK5 (5-CGUCUACCGA GAUCUGAAAtt-3), or GRK6 (5-GGACACAAAAGGAAUCAAGtt-3) (Ambion, Applied Biosystems, Warrington, UK) using the Interferin transfection reagent according to the manufacturer's instructions.

2.3. Assessment of agonist-stimulated Ca^{2+} signalling

To assess bradykinin mediated $[Ca^{2+}]_i$ changes, ULTR cells were seeded into 96-well plates. When 90% confluent, 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 bradykinin for various time periods. Agonist-stimulated fluorescence intensity changes were measured using a NovoStar imaging system (BMG Labtech, Aylesbury, UK), and plotted graphically as percentage increase over the fluorescence at time zero. To assess whether arrestin depletion affected bradykinin-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, NY, USA), as per manufacturers' instructions.

2.4. Determination of bradykinin-stimulated calcium signalling and receptor desensitisation in single cells

ULTR cells were seeded onto 25 mm glass coverslips and when at approximately 70% confluency, loaded with the calcium sensitive dye Fluo4-AM (3 µM) for 30 min at room temperature. Cells were maintained at 37 °C using a temperature controller and microincubator (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 min^{-1}. Images were captured using a Nikon (Surrey, UK) Eclipse C1Si confocal microscope, with an oil immersion 60× objective. $[Ca^{2+}]_i$ levels were determined by increases in cytosolic fluorescence in a defined area of interest exactly as described previously (Willets et al., 2005, 2008). Bradykinin B_2R desensitisation was determined using techniques similar to our previously validated protocols (Brighton et al., 2011b; Willets et al., 2009). Briefly, to assess receptor desensitisation cells were stimulated with a maximal agonist concentration (bradykinin 1 µM) for 30 s (termed R1), followed by a 5 min wash period before a second 30 s agonist challenge (100 nM, termed R2). Peak $[Ca^{2+}]_i$ R2 responses were significantly attenuated compared to R1 and the resulting reduction in the R2/R1 ratio is interpreted as an indication of receptor desensitisation (Willets et al., 2009). To determine whether arrestin proteins affected receptor desensitisation, cells were transfected with either negative control (100 nM), arrestin2 (100 nM) or arrestin3 (10 nM) siRNAs for 48 h prior to experimentation as described earlier.

2.5. Detection of MAPK activation

Agonist-driven ERK1/2 activity was detected using Western blotting techniques as described previously (Brighton et al., 2009). Agonist-stimulated p38 MAPK phosphorylation was also detected through a similar Western blotting approach (Brighton et al., 2011a). Briefly, ULTR cells were seeded into 6-well plates and grown to confluency. Cells were then serum-starved for 24 h prior to agonist addition. Signalling was terminated with the addition of lysis buffer (composition; 20 mM Tris–HCl (pH 7.4), 1% (v:v) Triton X-100, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanidate, 500 µM phenylmethanesulfonylfluoride, 0.1 mg ml^{-1} leupeptin, 0.2 mg ml^{-1} benzamidine, and 0.1 mg ml^{-1} pepstatin). Next, samples were centrifuged to clear insoluble material and an equal volume of 2× sample buffer (composition; 250 mM Tris–HCl, pH 6.8, 0.01% (w:v) bromophenol blue, 2% (w:v) sodium dodecyl sulphate, 40% (v:v) glycerol and 50 mM dithiothreitol) 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. Phospho-ERK1/2 was detected using a specific pERK1/2 antibody (Promega, Southampton, UK), which detects the dual phosphorylated P-loop pTEpY motif of ERK1/2; phospho-p38 MAPK (pTGpY) was detected using a specific pp38 antibody (Cell Signaling, Madison, WI, USA) followed by enhanced chemiluminescence detection as per manufacturers' instruction, and exposure to autoradiography film. Densitometric analysis of the resultant autoradiographs was undertaken using the GeneGnome image analysis system and software (Syngene, Cambridge, UK). 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,

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