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Functional interactions between the oxytocin receptor and the β_2-adrenergic receptor: Implications for ERK1/2 activation in human myometrial cells

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ABSTRACT

The Gq-coupled oxytocin receptor (OTR) and the Gs-coupled β_2-adrenergic receptor (β_2AR) are both expressed in myometrial cells and mediate uterine contraction and relaxation, respectively. The two receptors represent important pharmacological targets as OTR antagonists and β_2AR agonists are used to control preterm uterine contractions. Despite their physiologically antagonistic effects, both receptors activate the MAP kinases ERK1/2, which has been implicated in uterine contraction and the onset of labor. To determine the signalling pathways involved in mediating the ERK1/2 response, we assessed the effect of blockers of specific G protein-associated pathways. In human myometrial hTERT-C3 cells, inhibition of Gαi as well as inhibition of the Gαq/PKC pathway led to a reduction of both OTR- and β_2AR-mediated ERK1/2 activation. The involvement of Gαq/PKC in β_2AR-mediated ERK1/2 induction was unexpected. To test whether the emergence of this novel signalling mechanism was dependent on OTR expression in the same cell, we conducted experiments in HEK 293 cells that were transfected with the β_2AR alone or co-transfected with the OTR. Using this approach, we found that β_2AR-mediated ERK1/2 responses became sensitive to PKC inhibition only in cells co-transfected with the OTR. Inhibitor studies indicated the involvement of an atypical PKC isoform in this process. We confirmed the specific involvement of PKCζ in this pathway by assessing PKCζ translocation to the cell membrane. Consistent with our inhibitor studies, we found that β_2AR-mediated PKCζ translocation was dependent on co-expression of OTR. The present demonstration of a novel β_2AR-coupled signalling pathway that is dependent on OTR co-expression is suggestive of a molecular interaction between the two receptors.

1. Introduction

The oxytocin receptor (OTR) and the β_2-adrenergic receptor (β_2AR) are members of the large G protein-coupled receptor (GPCR) family. Both OTR and β_2AR are co-expressed in myometrial cells, but relay opposing signals of contraction and relaxation in these cells, respectively. While the OTR, is functionally coupled mainly to Gαq/11 as well as Gαi in myometrial cells, the β_2AR is predominantly a Gαs-coupled receptor and mediates uterine relaxation via an increase in intracellular cAMP levels [1]. The two receptors represent important pharmacological targets, because OTR antagonists and β_2AR agonists are used to control pre-term uterine contractions [2]. Despite their physiologically antagonistic effects, both receptors activate ERK1/2. Several studies, mostly performed in the rat myometrium, have identified the activation of ERK1/2 as a component of the cascade of events leading to the development of labor [3,4]. The onset of labor has been reported to be associated with basal activation of ERK1/2 [5].

The ERK1/2 signalling pathway constitutes one of the most ubiquitous signal transduction cascades. The mechanisms of ERK1/2 activation by GPCRs often depend on cell type and receptor involved, i.e. molecular context is an all-important determinant of the nature and duration of a particular ERK1/2 signal. Although both OTR and β_2AR activate ERK1/2, the mechanisms by which they do so are different. The OTR has been shown to couple to both Gαq and Gαi in myometrial cells. OTR activation of Gαq has been shown to be important for OT-stimulated phospholipase C (PLC) activation and elevation of

Abbreviations: AC, adenylyl cyclase; β_2AR, β_2-adrenergic receptor; cAMP, cyclic adenosine monophosphate; CFP, cyanide fluorescent protein; CYP, cyanopindolol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; DNA, deoxyribonucleic acid; EGF, epithelial growth factor; EGFR, epithelial growth factor receptor; ERK, extracellular signal-regulated kinase; ET-1R, endothelin-1 receptor; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HRP, horseradish peroxide; hTERT-C3, myometrial human telomerase reverse transcriptase-clone3 cells; ISO, isoproterenol; MAPK, mitogen activated protein kinase; OT, oxytocin; OTA, oxytocin receptor antagonist; OTR, oxytocin receptor; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase; PTX, *Pertussis* toxin; RNA, ribonucleic acid; YFP, yellow fluorescent protein.

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intracellular calcium in the myometrium and in the activation of ERK1/2 [6–8]. However, the OTR was also shown to activate Gαi in several cell types and OTR-mediated ERK1/2 phosphorylation has been reported to involve both PKC and Gαi-Gβγ pathways as well as transactivation of the epidermal growth factor receptor (EGFR) in PMH1 myometrial cells [8–11]. It has also been shown that the OTR interacts with the scaffolding protein β-arrestin2 [12] and that β-arrestin2 is involved in OT-mediated ERK1/2 activation [13,14]. Activation of ERK1/2 has been shown to be important in oxytocin-mediated myometrial contractions [15].

To date, most studies investigating β_2AR-mediated ERK1/2 activation have been performed in HEK 293 or COS-7 cells with transfected receptors. Some studies have suggested that β_2AR-mediated ERK1/2 activation involves Gβγ subunits, rather than the Gαs-cAMP-PKA pathway, which then act on a Ras-dependent pathway leading to activation of ERK1/2 [16]. Other studies show that β_2AR activates ERK1/2 via Gαs-PKA-dependent pathway [17]. Another G protein coupling mechanism relevant for ERK1/2 activation is the potential ability of β_2AR to undergo PKA-dependent phosphorylation in response to agonist, which leads to a loss of Gαs signalling and a switch to activation of Gαi [18]. Further, β_2AR-mediated ERK1/2 activation has also been reported to involve both G protein and β-arrestin components [19]. Other reports suggest that Src activation plays a role in β_2AR stimulation of ERK1/2 [20,21].

Previous studies have shown that β_2AR signalling could affect OTR signalling in the myometrium [22,23]. However, the mechanisms underlying crosstalk between the two receptors have not been investigated in detail. Unraveling the nature and consequences of OTR/β_2AR interactions and distinguishing direct physical interactions from indirect second messenger-mediated interactions, would be of significant pharmacological and physiological importance. The purpose of the present study was to define the signalling mechanisms involved in activating ERK1/2 by both the OTR and β_2AR in myometrial cells. Our studies demonstrate the involvement of several G proteins in OTR- and β_2AR-mediated ERK1/2 activation. We also identify a novel specific signaling pathway by which the β_2AR activates ERK1/2 in myometrial cells. This pathway involves Gαi-PI3K-PKCζ and is dependent on co-expression of the OTR and the β_2AR in the same cell. Taken together our results define a novel functional interaction between the two receptors which may, in turn, be based on a physical interaction.

2. Materials and methods

2.1. Reagents

Reagents were obtained from the following sources: DMEM/F12 tissue culture medium was from Invitrogen (Burlington, ON); fetal bovine serum (FBS) from Hyclone (Logan, UT); OT was from Sigma-Aldrich (St. Louis, MO); isoproterenol was from Tocris Bioscience (Bristol, UK); the inhibitors AG1478, wortmannin, PP2, PKCζ pseudosubstrate inhibitor, Gö6976 and Gö6983 were from Calbiochem (La Jolla, CA); *Pertussis* toxin (PTX) and Rö31-8220 and the protease inhibitors: leupeptin, benzamidine and trypsin were from Sigma-Aldrich (St. Louis, MO). Primary anti-phospho-ERK1/2 (T202, Y204) antibody was from Cell Signaling Technology (Beverly, MA); pan anti-ERK1/2 antibody was from Stressgen (Ann Arbor, MI). Secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG, was from Sigma-Aldrich (St. Louis, MO). Lipofectamine 2000 was from Invitrogen (Burlington, ON). All other analytical grade chemicals were obtained from Sigma-Aldrich, Fisher Scientific (Waltham, MA), or VWR (West Chester, PA). β-arrestin1/2 and control siRNA were obtained from Qiagen Inc. (Toronto, ON).

2.2. Constructs

OTR-YFP, an OTR construct with the coding sequence for YFP added in frame to the OTR C-terminus [24]. β_2AR-HA, was used as described previously [25]. PKC 1-GFP and PKCζ-GFP constructs were a kind gift from Dr S. Ferguson (Robarts Research Institute, London, ON). To generate mCherry-tagged PKCs, the constructs mentioned above and a pcDNA3.1(+)-mCherry vector were digested with *Nhe*I and *Bsr*GI to replace the GFP with mCherry. To generate the pcDNA3.1(+)-mCherry vector, pRSET-B-mCherry, a kind gift from Dr. Roger Y. Tsien (University of California, San Diego, CA), was amplified by PCR to introduce a NheI site in 5′ and KpnI site in 3′ of the mCherry and cloned into pcDNA3.1(+). shRNA constructs targetting β-arrestin1/2 were generous gifts from Dr. Marc Caron (Duke University, Durham, NC). All constructs were verified by bidirectional sequencing.

2.3. Cells culture and transfection

The myometrial human telomerase reverse transcriptase (hTERT)-HM cells were obtained from Dr. W. E. Rainey [26]. hTERT-C3 cells represent a selected subclone that we obtained by serial clonal dilution of hTERT-HM cells described previously [27]. hTERT-C3 cells were maintained in DMEM/F12 medium supplemented with 10% FBS and cultured at 37 °C in 5% CO_2. Cells close to confluency were passaged by trypsinization and plated in T175 flask at a one-quarter dilution every 4–5 days. HEK 293 cells were from Invitrogen (Carlsbad, CA) and were grown in Dulbecco's Modified Eagle's Medium/high glucose (DMEM) supplemented with 5% FBS and transfected using Lipofectamine 2000 as per manufacturer's instructions. Experiments were carried out 48 h after transfection.

2.4. Western blots

Immunoblotting was used to assess the expression of cellular proteins. Briefly, cells were plated onto culture dishes and grown in appropriate media to near confluency. Cells were starved for 24 h in either DMEM/F12 supplemented with only 0.5% FBS (hTERT-C3 cells) or DMEM/high glucose supplemented with only 0.5% FBS (HEK 293 cells). Cells were stimulated with ligand(s) for different times at 37 °C. Stimulation was stopped by two ice-cold PBS washes, and plates were flash frozen in liquid nitrogen. For inhibitor studies, cells were pre-treated with the inhibitor for 30 min to 1 h before stimulation with ligand(s). Cells were lysed on ice with Laemmli buffer (50 mM Tris–HCl [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, and 0.1 M β-mercaptoethanol) and were either homogenized using passage through a syringe and needle (hTERT-C3 cells) or by sonication (HEK 293 cells). Lysates were clarified by centrifugation at 15,000×*g* for 10 min at 4 C in a microcentrifuge. Proteins were denatured by boiling for 5 min, and subjected to SDS-PAGE and western blotting. Immunodetection involved different primary antibodies in conjunction with a secondary horseradish peroxidase-conjugated antibody and a chemiluminescence detection system (Supersignal; Pierce). Quantification of band intensities was performed using AlphaEase (Alpha Innotech Corp., San Leandro, CA).

2.5. Receptor quantification

Total receptor number for the β_2AR was calculated from binding experiments using [^{125}I]cyanopindolol (CYP) as the radioligand. Membranes were prepared and washed as previously described, with all steps of the process performed on ice [25]. Briefly, hTERT-C3 cells were washed twice with ice-cold PBS. They were then incubated for 15 min with ice-cold lysis buffer containing 15 mM Tris–HCl (pH 7.4), 0.3 mM EDTA, 2 mM $MgCl_2$, 5 μg/mL leupeptin, 10 μg/mL benzamidine and 5 μg/mL trypsin inhibitor. Subsequently, cells were collected and homogenized with a polytron. Lysates were centrifuged at 14,500 rpm for 20 min at 4 °C; the pellet was resuspended in ice-cold membrane buffer containing 50 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, 5 μg/mL leupeptin, 10 μg/mL benzamidine and 5 μg/mL trypsin inhibitor. Next, the pellet was homogenized using a 10-mL Potter-Elvehjem hand homogenizer and membranes were separated by centrifugation at 14,500 rpm