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Progesterone suppresses an oxytocin-stimulated signal pathway in COS-7 cells transfected with the oxytocin receptor

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ABSTRACT

The present study was conducted to determine if progesterone (P4) would inhibit oxytocin-stimulated phosphoinositide hydrolysis in COS-7 cells expressing transfected ovine oxytocin receptor (OTR) with little or no nuclear P4 receptor (nPR) protein present. The relative absence of nPR in these cells was confirmed by immunocytochemistry and RT-PCR. To investigate the effects of P4 on oxytocin (OT) signaling, cells were transiently transfected with the ovine OTR. Radioreceptor assay for [^{3}H]-OT binding confirmed the presence of a high affinity binding site for OT in transfected cells, while treatment with P4 and GTPγS (which uncouples the OTR from the heterotrimeric G-protein) increased the K_d for OT binding slightly. Cells were then assayed for inositol phosphate hydrolysis 48 h post-transfection. Pre-treatment of cells with P4 for 10 min significantly interfered with rapid (20 min) OT-stimulated inositol trisphosphate (IP_3) production. This inhibition was specific to P4, because pre-treatment of cells with promegestone (R5020), testosterone, mifepristone (RU 486), or cortisol did not decrease OT-stimulated IP_3 levels. By radioreceptor assay for PR, no measurable specific binding of R5020 was observed for either transfected or non-transfected cells. We conclude that P4 can inhibit OTR-mediated phosphoinositide hydrolysis in COS-7 cells that express little or no nPR protein. These data support a role for a non-genomic action for P4 in OTR signaling via some mechanism other than by binding to a membrane progestin receptor in an immortalized, transfected cell.

1. Introduction

It is commonly accepted that progesterone (P4) down-regulates the concentration of oxytocin receptors (OTRs) in the ovine uterus [1,2]. It was assumed that this was only through regulation of the OTR gene via a nuclear P4 receptor. However, Grazzini et al. [3] demonstrated that in rat uteri P4 can act non-genomically to interfere with the binding of oxytocin

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(OT) to its receptor. When these latter researchers transfected murine OTR into Chinese hamster ovary (CHO) cells, P4 was able to inhibit both OT binding and stimulation of inositol phosphate production by these cells [3]. Subsequently, it was reported that P4 interfered with the binding of OT to the OTR in ovine endometrial membranes [4] and suppressed OTR signaling in ovine endometrial explants [5]. The research of Dunlap and Stormshak [4] indicates that ovine endometrial plasma membranes are endowed with a high affinity binding site for progestins; presumably a putative membrane P4 receptor. These latter investigators also showed by competitive binding assays that P4 may be binding directly to the OTR or a closely associated protein; either a membrane-localized nuclear P4 or a novel membrane PR (mPR) similar to the G-protein coupled receptor reported to be present in seatrout ovaries [6]. An ovine mPR has recently been cloned, and is reportedly located in the ovine uterus as measured by RT-PCR [7]. Hence, it was reasoned that a cell line devoid of nuclear PR that was transfected with the OTR and was responsive to OT could be used to further examine the non-genomic action of P4 described above. The COS-7 cell line was chosen because these cells purportedly lack nuclear PR. Previous experiments by Riley et al. [8,9] indicated that the OTR would be functional when expressed in COS-7 cells, and treatment of transfected cells with OT would stimulate inositol phosphate production. A hypothesis for a mechanism of inhibition by P4 is that it interferes with the ability of the OTR to interact with the G-protein. To test this, GTPγS, a non-hydrolysable analog of GTP, was included in a radioreceptor assay to determine if it could mimic the effect of P4 on OT binding.

The present experiments utilizing COS-7 cells were conducted to determine if P4 inhibition of OT binding to the OTR and downstream signal transduction were due to an interaction of progestin with the OTR or a putative membrane PR.

2. Experimental

African green monkey kidney fibroblast cells transformed with SV40 antigen (COS-7) were maintained for all experiments in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/L D-glucose and L-glutamine (Invitrogen, Carlsbad, CA) and 10% fetal calf serum (FCS; Invitrogen). A human breast cancer adenocarcinoma epithelial cell line (MCF-7) was used as a positive control for RT-PCR experiments because these cells contain moderate levels of nuclear PR. These cells were maintained in Ham's F-12 DMEM with additives of sodium bicarbonate, L-glutamine (Invitrogen), and 10% FCS. COS-7 and MCF-7 cell lines were a generous gift of Dr. Frank Moore, Oregon State University.

A human mammary gland ductal carcinoma epithelial cell line (T47D) was obtained from the American Type Culture Collection (ATCC #HTB-133) and used as a positive control cell line for immunocytochemistry because these cells contain high levels of steroid hormone receptors. The T47D cells were maintained in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.2 units/mL bovine insulin and 10% fetal bovine serum.

2.1. *Experiment 1: OTR assay*

2.1.1. *Cell culture and transfection*

COS-7 cells were cultured in 90% DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C under 5% CO_2 in humidified air. One day prior to transfection, COS-7 cells were plated at a density of 5×10^5 cells/10 cm tissue culture dish. Ovine oxytocin receptor (oOTR) cDNA in mammalian expression vector (pCDNA3-OTR plasmid) at 4 μg/10 cm tissue culture dish was transiently transfected into COS-7 cells using the JetPEI transfection reagent (Polyplus transfection, Illkirch, France) according to the manufacturer's instructions. The oOTR was a generous gift from Dr. Thomas Spencer, Texas A&M University; see also Riley et al. [9].

2.1.2. *Membrane preparation*

Forty-eight hours after transfection, COS-7 cells were washed thrice with ice-cold PBS, collected by scraping, and pelleted at 500 × *g* for 5 min. Cold hypotonic lysis buffer (25 mM Hepes, pH 7.4, 2 mM EDTA, 0.1 mM PMSF, 1 μM pepstatin A, 10 μM leupeptin) was added to collected cells and incubated on ice for 15 min. Cells were lysed with 15 strokes of a Dounce homogenizer and the lysate centrifuged at 500 × *g*, 4 °C for 5 min to pellet nuclei and intact cells. The resulting supernatants were centrifuged at 200,000 × *g*, 4 °C for 30 min. The resulting crude membrane pellets were suspended in binding buffer (25 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.1 mM PMSF, 1 μM pepstatin A, 10 μM leupeptin). Protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA) with BSA as standard.

2.1.3. *Oxytocin receptor saturation isotherm binding assay*

Membranes at 0.5–1 μg protein/100 μL were resuspended in OT buffer (25 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.2% BSA). [^{3}H]-Oxytocin was purchased from PerkinElmer (Waltham, MA) and oxytocin from EMD Biosciences (LaJolla, CA). [^{3}H]-OT was diluted in the same OT buffer to 10× final concentrations. Binding assays (200 μL final volume) containing 0.5–1 μg cell membrane protein, increasing concentrations of ^{3}H-OT (0–20 nM) as indicated, 2.5 ng/mL P4, or 0.1% ethanol vehicle, or 100 μM GTPγS and OT buffer (total binding) or 2 μM unlabeled OT (non-specific binding) were incubated at 30 °C for 30 min. Binding assays were filtered though No. 32 glass-fiber filters (Whatman, Sanford, ME) on a Brandel Cell Harvester (Gaithersburg, MD), and washed three times with 5 mL ice-cold binding buffer. Radioactivity associated with membranes retained by glass filters was quantitated by liquid scintillation spectrometry on a Beckman LS 6500 scintillation counter (Fullerton, CA). Specific binding was calculated from total binding minus non-specific binding in the presence of excess (2 μM) oxytocin at each radioligand concentration. Affinity (K_d) and maximal binding capacity (B_{max}) values were obtained from saturation isotherm specific binding data by non-linear regression curve analysis using the standard equation for a rectangular hyperbola fitted to one-site with Prism 4 software (GraphPad Software Inc., San Diego, CA).