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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 [³H]-OT binding confirmed the presence of a high affinity binding site for OT in transfected cells, while treatment with P4 and GTP γ S (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 (IP₃) 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 IP₃ levels. By radioreceptor assay for PR, no measurable specific binding of R5020 was observed for either transfected or nontransfected 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 nongenomic action for P4 in OTR signaling via some mechanism other than by binding to a membrane progestin receptor in an immortalized, transfected cell.

Keywords

Oxytocin; Oxytocin Receptor; Progesterone; Nongenomic; Phosphoinositides

1. Introduction

It is commonly accepted that progesterone (P4) down-regulates the concentration of oxytocin receptors (OTR) in the ovine uterus [1,2]. It was assumed that this was only through regulation

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The authors have nothing to disclose.

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of the OTR gene via a nuclear P4 receptor. However, Grazzini et al. [3] demonstrated that in rat uteri P4 can act nongenomically to interfere with the binding of oxytocin (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 nongenomic 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, GTP γ S, 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 4,500 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% CO₂ 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 cDNA in mammalian expression vector (pCDNA3-OTR plasmid) at 4 μ g per 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 $500 \times g$ for 5 min. Cold hypotonic lysis buffer (25 mM Hepes, pH 7.4, 2 mM EDTA, 0.1 mM PMSF, 1 μ M pepstatin A, 10 μ M 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 $500 \times g$, 4°C for 5 min to pellet nuclei and intact cells. The resulting supernatants were centrifuged at $200,000 \times g$, 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 μ M pepstatin A, 10 μ M 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 μ g protein /100 μ l were resuspended in OT buffer (25 mM Tris, pH 7.4, 10 mM MgCl_2 , 0.2% BSA). [^3H]-oxytocin was purchased from Perkin Elmer (Waltham, MA) and oxytocin from EMD Biosciences (LaJolla, CA). [^3H]-OT was diluted in the same OT buffer to 10 X final concentrations. Binding assays (200 μ l final volume) containing 0.5 ~ 1 μ g cell membrane protein, increasing concentrations of ^3H -OT (0 to 20 nM) as indicated, 2.5 ng / ml P4, or 0.1 % ethanol vehicle, or 100 μ M GTP γ S and OT buffer (total binding) or 2 μ M unlabeled OT (nonspecific binding) were incubated at 30°C for 30 min. Binding assays were filtered through 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 μ M) 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).

2.2 Experiment 2. Inhibition of downstream OTR signaling by P4

2.2.1 Transfections—COS-7 cells were grown to 100 % confluence (1×10^6 to 2×10^6 cells/ plate) in 10 cm plates ($n = 5$) in 12 ml DMEM. Cells were transfected with 24 μ g/ plate of a pcDNA3 plasmid containing the cDNA sequence for the ovine oxytocin receptor. The plasmid DNA was inserted into cells by treatment with 60 μ l/ plate Lipofectamine 2000TM (Invitrogen) in 3 ml/ plate of Opti-MEM[®] reduced serum medium (Invitrogen). Cells were assayed for response to OT stimulation 48 h after transfection by measuring changes in phosphoinositide hydrolysis.

2.2.2 Measurement of Inositol Phosphate Accumulation—Cells (approximately 100,000/ well) were plated into 24 well plates 24 h after transfection and allowed to attach overnight. Once attached 2 μ Ci/ ml myo-[2- ^3H] inositol (18.5 Ci/ mmol, PerkinElmer Life and Analytical Sciences, Shelton, CT) were added in 0.5 ml serum- and inositol-free 20 mM Hepes-DMEM (H-DMEM, pH 7.4; Invitrogen) and allowed to incubate at 37°C in a humidified atmosphere overnight. The following day (48 h after transfection of cDNA), cells were assayed for response to OT in the following manner:

Wells were allotted to a 2 x 2 factorial arrangement of treatment groups with two dosages of P4 (0 and 2.5 ng/ ml [5]; Steraloids, Newport, RI) and two dosages of OT (0 and 100 nM [9]; Calbiochem, San Diego, CA). Incubation was begun by a 0.5 ml media change to H-DMEM containing 10 mM LiCl (to prevent dephosphorylation of inositol species) and vehicle, or LiCl and P4 followed by preincubation of cells at 37°C for 10 min. Vehicle or OT was then added and incubation continued for an additional 20 min, after which time all media were removed,

and 0.5 ml 5 % ice-cold trichloroacetic acid (TCA) was added to wells. The TCA solutions were collected and after extraction three times with 2 ml diethyl ether they were stored at 4° C until analyzed for inositol mono-, bis-, and trisphosphate (IP, IP₂, and IP₃, respectively) content by chromatographic separation. Total lipid incorporation of myo-[2-³H] inositol (not converted into inositol species) was determined by addition to wells of 0.5 ml of 1N NaOH to solubilize membranes and 0.5 ml of 1N HCl to neutralize alkalinity. Inositol phosphate species in extracted TCA solutions were separated by a chromatographic procedure originally reported by Mirando et al. [10] and further described by Bishop and Stormshak [5] utilizing increasing molar concentrations of ammonium formate buffer for elution of multiple phosphorylated inositol species in a step-wise manner. Radioactivity of inositol species was determined by liquid scintillation spectrometry. Data on incorporation of [³H]-inositol into IP, IP₂, and IP₃ are expressed as % DPM [³H]-IP_n / total [³H]-inositol per well.

2.3. Experiment 3. Specificity of inhibition of OTR signaling

COS-7 cells were maintained as in Exp. 2 until 50–70% confluence (approximately 5 x 10⁵ to 14 x 10⁵ cells/ plate; n = 4). Cells were then transfected by addition of 7 µg/ plate OTR plasmid using 14 µl/ plate JetPEI™ reagent (Qbiogene, Morgan Irvine, CA) in 1 ml/ plate of 150 mM NaCl. The JetPEI™ reagent was used for the remainder of the experiments due to observed lower cytotoxicity of this reagent versus Lipofectamine 2000™. Cells were again assayed for response to OT stimulation 48 h after transfection by measuring changes in phosphoinositide hydrolysis. Plates were pre-incubated in [³H]-inositol as described for Exp. 2. Wells were then allotted to treatment groups of vehicle, P4 (2.5 ng/ ml), R5020 (2.5 ng/ ml; promegestone, a synthetic progestin; PerkinElmer Life and Analytical Sciences), RU 486 (2.5 ng/ ml; mifepristone, an antagonist of the classical nPR, included as a negative control; Sigma-Aldrich, Saint Louis, MO), testosterone (2.5 ng/ ml; Sigma-Aldrich), and cortisol (2.5 ng/ ml; Sigma-Aldrich). Treatments were added to medium containing LiCl, and plates were incubated as described for Exp. 2. All wells were challenged with OT (100 nM), and effect of steroids on IP₃ production in response to OT was determined as described for Exp. 2.

2.4 Experiment 4. Presence of nPR in COS-7 cells

2.4.1. Immunocytochemistry—COS-7 cells at 100 % confluence (1 x 10⁶ to 2 x 10⁶ cells/ plate; n = 1) were harvested by trypsin lysis with 3 ml TripLE® Express reagent (Invitrogen) and transferred in 2 ml DMEM to 4 chamber slides (0.5 ml/ well, n = 4). Cells were allowed to attach overnight and slides were snap frozen in liquid N₂ before evaluation for the presence of genomic progesterone receptor isoforms A and B (PR-A and PR-B), estrogen receptor, and androgen receptor by immunocytochemistry. Anti-PR (Ab-8, Neomarker Inc., Fremont CA) was utilized at a concentration of 0.1 µg/ ml, anti-ER (1D-5, Biogenex, San Ramon, CA) was utilized at a dilution of 1:50, and anti-AR (F-39, Biogenex) was also utilized at a dilution of 1:50. Immunocytochemical analysis was performed as cited before [11,12].

2.4.2. Reverse-Transcription PCR for nuclear PR—COS-7 and MCF-7 cells were harvested at 100 % confluence (1 x 10⁶ to 2 x 10⁶ cells/ plate) and processed for mRNA isolation by RNeasy kit (small scale phenol-free total RNA isolation kit, Ambion Austin, TX). Reverse transcription PCR (RT-PCR) was performed by adding isolated mRNA (0.34 µg/ sample) as template to Super-Script One-Step RT-PCR with Platinum Taq (Invitrogen) with primers 5'CCACAGGAGTTTGTCAGCT3' (forward) and 5'CGGGACTGGATAAATGTATT3' (reverse; Invitrogen) based on published cDNA sequence of African green monkey nPR (NCBI database accession number S71038). Synthesis and PCR amplification of cDNA (n = 4) were performed in a volume of 50 µl using 35 cycles of PCR. Products (25 µl) were then loaded onto a 2 % agarose gel for electrophoresis at 100 V for 1 h, and subsequently the gel was stained with Syber Green Stain (Invitrogen) to visualize amplified cDNA.

2.5. Experiment 5. Presence of progestin binding sites

COS-7 cells were maintained in 15 cm plates (approximately 5×10^5 to 14×10^5 cells/ plate) in 30 ml DMEM and either transfected with 10 μ g/ plate oOTR plasmid ($n = 3$) with 20 μ l/ plate Jet-PEI in 2 ml/ plate of 150 mM NaCl, or remained non-transfected ($n = 3$). Cells were subjected to a full media change at 24 h post-transfection and were harvested 48 h post-transfection via scraping with ice cold phosphate buffered saline (pH 7.0, PBS) containing 1X protease inhibitor cocktail (Calbiochem, San Diego CA). Cells were then subjected to R5020 binding assay.

2.5.1 R5020 Binding Assay—Cells in cold PBS were transferred to a ground glass homogenizer. Cells were homogenized by 10 passes of the pestle and the homogenate was transferred to centrifuge tubes. Cellular debris was pelleted at 1000 x g for 10 min, and the supernatant was then subjected to centrifugation at 100,000 x g to recover membranes. Protein content of membrane fraction was then analyzed by use of the BCA Assay Kit (Pierce Biotechnology, Inc. Rockford, IL) and adjusted to 1 mg protein/ ml by addition of 25 mM Tris-HCl, 0.01 % NaN_3 (pH 7.4).

Membranes were evaluated for specific binding of progestin as described by Dunlap and Stormshak [4]. While gently vortexing, 100 μ g aliquots of the membrane preparation were added to four tubes to permit individual analyses in duplicate. To two tubes [^3H]-R5020 (87 Ci/ mmol, Perkin Elmer Life and Analytical Sciences) was added in concentrations ranging from 0.5 to 10 nM, and 2.5 μ l of absolute ethanol (vehicle). These tubes containing the sample, vehicle, and labeled ligand were utilized to estimate total binding of [^3H]-progestin. To the remaining two tubes was added the labeled ligand (in 2.5 μ l absolute ethanol) plus a 200-fold excess of unlabeled ligand (in 2.5 μ l absolute ethanol) for the purpose of determining nonspecific binding. The volume of all tubes was adjusted to 155 μ l by addition of 50 μ l of TBM buffer (25 mM Tris-HCl, 0.01 % NaN_3 , 0.2 % BSA, 20 mM MnCl_2 , pH 7.4). All tubes were gently vortexed and then incubated for 18 h at 4° C.

Subsequently, all tubes were placed into an ice bath and to each was added 750 μ l TBM buffer (4° C) and 1 ml of pelleting buffer (25 mM Tris-HCl, 0.01 % NaN_3 , 40 % [wt/ vol] polyethylene glycol; pH 7.4, 4° C). All tubes were then vortexed and centrifuged at 2000 x g for 15 min at 4° C. The supernatant was then decanted and the tubes were inverted for 10 min at 4° C to drain away excess buffer. The remaining pellet was then resuspended in 1 ml each of TBM and pelleting buffer, vortexed, and centrifuged as above. The supernatant was again decanted and the tubes inverted to drain for 10 min at 4° C. The pellet was finally resuspended in 500 μ l of 25 mM Tris - HCl, 0.01 % NaN_3 (pH 7.4) and decanted into vials containing 5 ml of CytoScint (ICN Biomedicals, Inc., Irvine, CA) for liquid scintillation analysis. Vials were vortexed, shielded from light for 12 h, and then counted. Specifically bound progestin for each concentration used was determined by subtracting DPM [^3H]-R5020 nonspecifically bound from DPM [^3H]-R5020 total bound.

2.6. Statistics

Data on production of IP, IP₂, and IP₃ were analyzed statistically as a randomized complete block design by use of a two-way or one-way ANOVA for Exp. 2 and Exp. 3, respectively. Differences among means were determined by use of the general linear model (GLM) procedure of SAS when appropriate.

3. Results

3.1. Experiment 1

Transfection of oOTR plasmid into COS-7 cells resulted in high affinity binding sites for OT in these cells (Table 1 and Figure 1). Addition of 2.5 ng/ml P4 increased the K_d for OT binding slightly, as well as the B_{max} . These effects of P4 are similar to those observed by the addition of 100 μ M GTP γ S, which has been shown to uncouple the receptor from the G-protein [13]. These cell responses to P4 and GTP γ S are considered to be minor and non-significant.

3.2. Experiment 2

Addition of 2.5 ng/ml P4 to medium containing LiCl during pre-incubation significantly suppressed OT-stimulated inositol trisphosphate (IP₃) and inositol bisphosphate (IP₂) production in cells transfected with the ovine OTR (IP₃, P4 x OT interaction $P < 0.03$, Figure 2; IP₂, P4 x OT interaction $P = 0.0001$, Figure 3). These data correlate with the results of Bishop and Stormshak [5] who observed P4 inhibition of OT-induced IP₂ and IP₃ production in explants of endometrium of proestrous ewes. However, while there was a significant P4 x OT interaction ($P < 0.02$, Figure 4), due to the high amount of variation between separate transfections there was no significant decrease in OT-induced IP production by pre-incubation with P4. Nevertheless, there was a tendency for P4 to reduce the amount of IP produced in response to OT.

3.3. Experiment 3

The inhibition of OT-stimulated IP₃ production appears to be specific to P4 because pre-treatment with similar dosages of testosterone, cortisol, mifepristone (RU 486), and promegestone (R5020) did not significantly inhibit IP₃ production by OT in OTR transiently transfected cells (Figure 5). Testosterone and RU 486 did not increase levels of IP₃ in transfected cells in the absence of OT (data not shown).

3.4. Experiment 4

There was no significant staining for the nuclear progesterone, nuclear estrogen, or nuclear androgen receptor above background levels in COS-7 cells (Figure 6). However, robust staining for all three receptor isoforms was observed in the positive control cell line T47D cells (human breast carcinoma). The results of the ICC were confirmed by RT-PCR, which revealed little mRNA for nPR present in COS-7 when compared to the control MCF-7 cell line (data not shown).

3.5. Experiment 5

There was no measurable specific binding of the synthetic progestin R5020 to membrane fractions of both transfected and non-transfected COS-7 cells (Figure 7). Because there was no specific binding of progestin, [³H]-progesterone was also tested at a concentration of 5 nM, and the same results were obtained (no measurable specific binding; data not shown).

4. Discussion

These experiments provide evidence that the inhibitory effect of P4 on the ovine OTR is specific to P4, and is probably not mediated by the classical nPR, or the novel heptahelical mPR. Other researchers have demonstrated that COS-7 cells do express very low levels of nPR, but lack the necessary co-factors to induce transcriptional effects through nPR [14].

The exact mechanism of inhibition of the OTR by P4 is still not known. Because there is no measurable specific binding of progestin to membranes of transfected COS-7 cells, P4 may be

interacting with proteins that block the receptor's ability to interact with G-proteins. However, the present data do not support such a mode of action by P4. Alternatively, P4 may be directly interfering with other processes that are critical to allow the OTR to interact with G-proteins such as dimerization of the OTR or translocation of the OTR to lipid rafts. It is important to note that Dunlap and Stormshak [4] observed specific and saturable binding of progesterone and R5020 to ovine endometrial membrane fractions in which P4 inhibited the binding of OT to the OTR. They also observed that the binding of R5020 could be successfully competed for by 200-fold excess of unlabeled OT when tested in a radioreceptor exchange assay performed on ovine endometrial membranes [4]. Based upon those data, it was suggested that a protein closely associated with the OTR or the OTR itself contains the binding site for P4. Although there was no significant effect of R5020 on IP₃ production by OT-stimulated COS-7 cells, the binding of R5020 to COS-7 cell membranes was investigated to ascertain if the membrane environment of the COS-7 cell is similar to ovine endometrial membranes [4]. Because there is a lack of specific, measurable binding of progestins to the COS-7 cell membranes it appears that the two membrane environments do differ with respect to their protein content. Therefore, the mechanism of P4 inhibition of OT binding to, and signal propagation through, the ovine OTR *in vivo* may be slightly different than that which occurs in the transfected cellular system investigated here. Nevertheless, progestin inhibition of OTR response to OT may be of physiological significance during early gestation in the sheep. The developing ovine conceptus synthesizes P4 which could act locally within the uterus to suppress binding of OT to its receptor, thus ensuring uterine quiescence and embryo survival.

It is noteworthy that P4 suppressed the ability of OT to stimulate phosphoinositide hydrolysis in two different types of OTR-transfected cells (COS-7 in the present experiment and CHO as reported by Grazzini et al. [3]) as well as ovine endometrial explants [5]. By simply comparing the suppressed responses of these transfected cells and endometrial explants to OT after exposure to P4, one might assume existence of an identical mechanism of inhibition by P4. However, while high affinity membrane binding sites for progestins were detected in transfected CHO cells [3] and ovine endometrium [4] none were detected in transfected COS-7 cells. It is conceivable that CHO cells (being of reproductive tract origin) and ovine endometrium may contain a progestin-sensitive membrane OTR binding partner that is lacking in kidney derived COS-7 cells. Grazzini et al. [3] also observed a large decrease in binding capacity of the murine OTR in CHO cells when incubated with 0.1 and 10 μ M P4, whereas in the investigated system, use of 8 nM P4 did not have a dramatic effect on OT binding capacity. Although a decrease in OTR signaling was observed (via a decrease in IP₃ production), in order to detect any inhibition of binding capacity a greater dosage of P4 may have to be investigated. The authors declined to perform these experiments in this system to keep the dosages investigated near low, physiologic levels.

In ovine OTR-transfected cells P4 was the only steroid or progestin investigated that was able to significantly inhibit inositol phosphate hydrolysis stimulated by OT at physiologically relevant levels. The mechanism by which P4 is able to inhibit OT-stimulated responses *in vivo* is still unknown. The present data serve to underscore the potential for error when commonality of response to a hormone by transfected cells and normal target cells of the animal is assumed to always occur by the same mechanism of action of the hormone.

Collectively, it appears that the mechanism by which P4 can inhibit OT-induced responses may differ among cell type. In COS-7 cells, bearing transfected OTR and devoid of high affinity binding sites for progesterone, this steroid is still apparently able to somehow interfere with oxytocin stimulation of the phosphoinositide cascade.

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Figure 1.

(A and B): Saturation isotherm binding assays with $[^3\text{H}]$ -oxytocin were conducted on membranes from ovine oxytocin receptor-transfected COS-7 cells. Shown is (A) specific binding or (B) scatchard transformation of specific binding for samples including 100 μM GTP γ S (closed triangles, dashed line), 2.5 ng/ml progesterone (closed squares, dotted line), or 0.1 % ethanol vehicle (open circles, solid line). Non-specific binding for each concentration of radioligand was defined by the inclusion of 100 nM oxytocin and was less than 20% of total binding.

Figure 2.

Effect of P4 on OT-stimulated IP₃ production in transfected COS-7 cells (means $\pm$ SE; n = 5 plates, 6 wells/treatment). Treatment with OT markedly increased IP₃ produced by COS-7 cells (*control vs OT only, $P < 0.002$). However, P4 significantly interfered with OT-induced IP₃ production (**OT+P4 vs OT only, $P < 0.03$).

Figure 3. Effect of P4 on OT-stimulated IP₂ production in transfected COS-7 cells (means $\pm$ SE; n = 5 plates, 6 wells/treatment). Treatment with OT markedly increased IP₂ produced by COS-7 cells (*control vs OT only, $P < 0.0001$). However, P4 interfered with OT-induced IP₂ production (**OT+P4 vs OT only, $P = 0.0001$).

Figure 4.

Effect of P4 on OT-stimulated IP production in transfected COS-7 cells (means $\pm$ SE; $n = 5$ transfected plates, 6 wells/treatment). Treatment with OT markedly increased IP produced by COS-7 cells (*control vs OT and control vs OT+P4, $P < 0.0001$). While there was no significant decrease of OT-stimulated IP by pre-treatment with P4 there was a significant interaction between P4 and OT (OT $\times$ P4 interaction by 2-way ANOVA, $P < 0.02$). This was due to a tendency for P4 to reduce the absolute levels of IP produced by OT on a plate - to - plate basis.

Figure 5. Effect of steroids on mean ($\pm$ SE) OT-stimulated IP₃ production in transfected COS-7 cells (n = 4 transfected plates, 4 wells/ treatment). Only P4 was able to significantly inhibit OT-stimulated IP₃ production (*OT only vs P4+OT, P = 0.001).

Figure 6. ICC results of both COS-7 and T47D cells for nuclear steroid hormone receptors. COS-7 cells did not exhibit any significant staining above background for any of the receptors.

Figure 7.

Results of binding of [³H]-R5020 to both ovine oxytocin receptor (oOTR) transfected (n = 3 transfected plates) and non-transfected (n = 3 plates) COS-7 cells. There was no measurable specific binding of R5020 detected in either transfected or non-transfected COS-7 cells. NSB, nonspecifically bound.

Table 1

Dissociation rate constant (K_d) for [^3H]-oxytocin binding and maximal number of transfected ovine oxytocin receptors (B_{max}) in COS-7 cell membranes^a

Item	Treatment ^b		
	Control	+ P4	+ GTP γ S
K_d (nM)	6.6 ± 0.9	7.5 ± 0.6	8.8 ± 0.7
B_{max} (fmol/mg protein)	226 ± 13	232 ± 7	226 ± 8

^a means $\pm$ SE for three replicates of each treatment regimen.

^b Transfected COS-7 cells were incubated in the absence (control) or presence of 2.5 ng/ml progesterone (P4) or 100 μM GTP γ S