Oestradiol is a potent mitogen and modulator of GnRH signalling in αT3–1 cells: are these effects causally related?

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Abstract

GnRH acts via phospholipase C (PLC) activating G-protein coupled receptors to stimulate secretion of gonadotrophins from gonadotrophs. These cells are also regulated by gonadal steroids, which act centrally to influence GnRH secretion, and peripherally to modulate GnRH action. We have shown that oestradiol can stimulate proliferation and modulate GnRH-stimulated [3H]inositol phosphate ([3H]IPx) accumulation (used as a measure of PLC activity) in a gonadotroph-derived cell line (αT3–1). Here we show that when αT3–1 cells were incubated in medium with 2% foetal calf serum (FCS), [3H]thymidine incorporation was not stimulated by 10 or 1000 nM raloxifene was reversed competitively by oestrogen antagonist, raloxifene. The inhibitory effect of oestradiol but was reduced to <2% of control by the effects are best revealed in the presence of raloxifene. Using this strategy, we found that cells cultured for 4 days with 10 nM raloxifene expressed GnRH receptors (Kd for 125I-buserelin 4·33 nM) and that their activation by GnRH caused a concentration-dependent increase in [3H]IPx (in cells labelled with [3H]inositol) and inositol 1,4,5 trisphosphate (in unlabelled cells). Addition of 10 nM oestriadiol (to overcome receptor blockade by raloxifene) reduced GnRH receptor number by 31% but increased maximal effects on [3H]IPx and Ins(1,4,5)P3 approximately 4-fold. The effects of oestriadiol on GnRH receptor number and signalling were not, however, mimicked by culture for 2 days in medium with 10% FCS and the S-phase blocker, thymidine (15 mM). This treatment increased the proportion of cells in the S-phase 2– to 3-fold but did not alter GnRH receptor number or signalling. Other treatments which altered cell cycle transition (hydroxyurea, colcemid, methotrexate) also failed to alter GnRH receptor number or signalling and no correlation was seen between GnRH receptor number or GnRH-stimulated [3H]IPx accumulation and the proportion of cells in the S-phase or G2/M-phases of the cell cycle. Thus, oestradiol has pronounced effects on GnRH signalling, proliferation and cell cycle distribution in αT3–1 cells, but these trophic effects do not underlie the modulation of GnRH signalling.


Introduction

Gonadotrophin-releasing hormone (GnRH) acts via phospholipase C (PLC) activating G-protein coupled receptors on pituitary gonadotrophs (Kakar et al. 1992, Tsutsumi et al. 1992, Sealfon et al. 1997) to regulate the release of the gonadotrophins, luteinising hormone (LH) and follicle-stimulating hormone (FSH). The GnRH-stimulated hydrolysis of phosphoinositides generates diacylglycerol, which activates some isofoms of protein kinase C (PKC), and inositol 1,4,5 trisphosphate (Ins(1,4,5)P3), which mobilises Ca2+ from intracellular stores (Conn 1995, Stojilkovic & Catt 1995, Kratzmeier et al. 1996). GnRH also increases Ca2+ entry into gonadotrophs, via voltage-operated Ca2+ channels, and the increase in cytosolic Ca2+ ion concentration caused by accelerated entry and mobilisation is primarily responsible for the increase in exocytosis (Hansen et al. 1987, McArdle...
Gonadotrophs are also influenced by gonadal steroids, which act centrally to control GnRH secretion and also directly at the pituitary to modulate gonadotroph responsiveness to GnRH. The direct effects have been extensively studied in primary cultures of rat pituitary cells in which oestriadiol can amplify GnRH-stimulated gonadotrophin secretion (Tang et al. 1982) and can increase GnRH receptor number (Clayton 1989) and influence gonadotrophin subunit expression (Ramey et al. 1987, Phillips et al. 1988). In terms of GnRH signalling, oestradiol has been shown to influence the Ca\(^{2+}\) response to GnRH (Leong & Thorner 1991) and the effects of GnRH on Ca\(^{2+}\) mobilisation and Ins(1,4,5)P\(_3\) generation are increased in cells from pro-oestrous rats and reduced in those from ovariectomised rats (Mitchell et al. 1988). Moreover, oestradiol has been shown to increase phorbol ester-stimulated gonadotrophin secretion and anterior pituitary levels of PKC (Drouva et al. 1990). Thus, although the mechanisms of oestrogen action on gonadotrophs are not well defined, it appears to modulate GnRH action by exerting coordinated effects at, and distal to, the GnRH receptor. To further investigate the mechanisms involved, we have explored the steroidal regulation of the gonadotroph-derived αT3–1 cell line, which expresses GnRH receptors and the glycoprotein hormone α-subunit, but does not express the β-subunit of LH or FSH (Windle et al. 1990, McArdle et al. 1992). Oestradiol stimulates the proliferation of these cells (as measured by increased cell number and \(^{3}H\)thymidine incorporation), reduces GnRH receptor number and increases GnRH-stimulated accumulation of \(^{3}H\)inositol phosphate (\(^{3}H\)IP\(_3\)), an index of PLC activity measured in cells labelled with \(^{3}H\)inositol and stimulated in the presence of LiCl (McArdle et al. 1992, Ortmann et al. 1995). Interestingly, the oestradiol receptor antagonist, raloxifene, inhibited the proliferation of αT3–1 cells cultured in phenol red-free medium with steroid-stripped sera (McArdle et al. 1992), implying that a low residual level of oestrogenic activity was stimulating their proliferation and that the full extent of oestradiol’s effects had therefore been underestimated.

Oestradiol is an important mitogen for many cell types, although its effects are probably best characterised for breast cancer cell lines. In MCF-7 cells, for example, oestradiol has a pronounced proliferative effect reflecting its ability to regulate the expression and/or activity of a number of genes (e.g. thymidine kinase, myc, calmodulin, cyclin B1) which influence transit through the cell cycle (Thomas & Thomas 1994). In addition, the synthesis of oestrogen receptors in these cells occurs preferentially during the G0/G1 transition (Rostagno et al. 1996) implying that the action of oestrogen is both dependent upon, and a determinant of, cell cycle kinetics. This reciprocal relationship is also seen for the PLC/Ca\(^{2+}/PKC\) signalling pathway, which in many systems influences cell cycle kinetics but is also dependent upon cell cycle stage. Thus, for example, Ins(1,4,5)P\(_3\) levels and Ca\(^{2+}\) signalling can vary dramatically through the cell cycle (Ciapa et al. 1994) just as activation of PLC (via G-protein coupled receptors) can stimulate cell proliferation and alter cell cycle progression (Smith et al. 1989, Noh et al. 1995, Honma et al. 1996, Sun et al. 1997). In the light of such observations it is plausible that GnRH signalling is influenced by cell cycle stage in αT3–1 cells and that the effects of oestradiol on GnRH signalling are due to its effects on cell cycle distribution. Since αT3–1 cells have a phenotype similar to that of immature gonadotrophs (GnRH receptor and gonadotrophin α-subunit expression without β-subunit expression) we were particularly interested in the possibility that such effects might be pertinent during foetal or neonatal development (when proliferation rates are high). To test these possibilities, we have defined the influence of oestradiol in αT3–1 cells, exploiting competitive antagonism of oestrogen receptors to reveal the full extent of oestradiol’s effects but have found no causal relationship between its effects on proliferation and GnRH receptor signalling.

Materials and Methods

Culture media, sera and lipofectin were obtained from Gibco BRL (Paisley, Strathclyde, UK) and culture dishes were from Gibco BRL or Falcon (Becton Dickinson, Oxford, UK). The αT3–1 cells, raloxifene and buserelin or \(^{125}\)I-buserelin (D-Ser((tBu))\(^{6}\),Pro-NHEt)GnRH, at 2000 Ci/mmol were kindly provided by Dr P Mellon (University of California, San Diego, CA, USA), M Neidenthal (Eli Lilly, IN, USA) and Drs Sandow and Von Rechenberg (Hoechst Marion Roussel, Frankfurt, Germany) respectively. GnRH and pituitary adenylate cyclase-activating poly peptide 1–27 (PACAP27) were from Calbiochem (Nottingham, Notts, UK), \(^{3}H\)thymidine (70–86 Ci/mmol), d-myo-[\(^{3}H\)]inositol 1,4,5 tris-phosphate (20–60 Ci/mmol), myo-[2,\(^{3}H\)]inositol (80–120 Ci/mmol) were from Amersham International plc (Little Chalfont, Bucks, UK) and flow cytometry materials were obtained from Becton Dickinson Diagnostic Systems. The oestrogen–response element (ERE) reporter (vit-ERE-luciferase) was prepared as described previously (Pennie et al. 1998) by cloning two tandem copies of the ERE from the vitellogenin gene upstream of the minimal SV40 promoter driving a luciferase gene (pGL3; Promega, Southampton, Hants, UK) and the pGL3 plasmid alone was used for normalisation. Luciferase assay reagents were obtained from Promega, Meltilex wax scintillation strips and Wallac A harvesting filters were obtained from Wallac (Turku, Finland) and the 96-well plate harvester was from Tomtec (Orange, CT, USA). Mr D Barrow (Bakers of

Nailsea, Nailsea, North Somerset, UK) kindly supplied the bovine adrenal glands. All other reagents were from standard commercial suppliers.

**Cell culture**

αT3-1 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 5% horse serum, 2 mM l-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. They were incubated at 37 °C in water-saturated air with 5% CO₂ and passed weekly after trypsinisation (500 µg/ml trypsin, 200 µg/ml EDTA). Twenty-four hours after plating in normal culture media, 17β-oestradiol and anti-oestrogen was added to the cultures, as indicated in the legends, for 2- or 4-day pre-treatments. Normal culture media was used in the 2-day pre-treatments and a low FCS culture medium, containing no horse serum and only 2% charcoal-treated FCS, was used for the 4-day pre-treatments. The charcoal-treated FCS was prepared by incubation with charcoal-dextran T-70 for 25 min (25 mM Tris/1 mM EDTA, 500 mg/l dextran T-70, 5 g/l activated charcoal), a procedure which reduces the treatments. The charcoal-treated FCS was prepared by incubation with charcoal-dextran T-70 for 25 min (25 mM Tris/1 mM EDTA, 500 mg/l dextran T-70, 5 g/l activated charcoal), a procedure which reduces the oestrogen was added to the cultures, as indicated in the legends, for 2- or 4-day pre-treatments. Normal culture media was used in the 2-day pre-treatments and a low FCS culture medium, containing no horse serum and only 2% charcoal-treated FCS, was used for the 4-day pre-treatments. The charcoal-treated FCS was prepared by incubation with charcoal-dextran T-70 for 25 min (25 mM Tris/1 mM EDTA, 500 mg/l dextran T-70, 5 g/l activated charcoal), a procedure which reduces the Oestradiol concentration of FCS to >10⁻¹¹ M (McArdle et al. 1992)

**Measurement of cell number and [³H]thymidine incorporation**

Cell number was determined by DNA measurement. Cell pellets or cells in culture plates were washed in PBS and lysed by freezing, thawing and trituration in 1 ml 100 mM Tris, 1·0 mM NaCl, 10 mM EDTA. Two hundred and fifty microlitres of the cell lysate were then incubated at 21 °C with 4 µg/ml bisbenzimide (Hoechst 33258 trihydrochloride, Hoechst UK Ltd, Hounslow, Middlesex, UK). DNA content was determined by reading fluorescence at 492 nm against a calf thymus DNA standard curve (Holtorf et al. 1989). Fluorescence of 1 µg/ml DNA standard was equivalent to 9·9 × 10⁴ αT3-1 cells. Incorporation of [³H]thymidine into newly synthesised DNA was used as an index for proliferation. Between 3 and 5 × 10⁴ cells/cm² growing area were cultured with the appropriate treatment(s) in 96-well plates in 250 µl media. [³H]thymidine (0·025 µCi in 10 µl RPMI 1640 medium (Gibco)) was added to each well for the final 6 h of incubation. After a single wash with pre-warmed Hank’s buffered saline solution the cells were incubated for 10 min at 37 °C with 150 µl trypsin/EDTA solution in each well. The cells were harvested onto Wallac filters using a 96-well plate harvester. A Meltilex A wax scintillant strip was melted onto the dried filter and [³H]thymidine incorporated was then determined by β-counting and expressed as [³H]thymidine incorporated over 6 h per million cells. Cell number was estimated by DNA measurement of cells in 24-well plates grown concurrently under the same conditions and at the same seeding density (i.e. cells per cm² of growth area).

**Determination of oestrogen receptor activation (trans-activation of vit-ERE-luciferase)**

Oestrogen receptor activity was determined using cells transiently transfected with a vector containing the vitellogenin ERE upstream of the minimal SV40 promoter (pGL3) driving luciferase expression. In parallel, cells were transfected with the control pGL3 luciferase reporter alone. Cells were transfected by incubating for 6 h (37 °C) in OptiMEM (Gibco) and 5 µg/ml lipofectin with either 0·5 µg/ml vit-ERE-luciferase or 1·0 µg/ml pGL3. After transfection, the OptiMEM/lipofectin/DNA was replaced with DMEM and oestradiol and/or raloxifene, as required. After 4 days the cells were washed with PBS, lysed, and centrifuged (12 000 g, 2 min). The supernatant was then frozen (–70 °C) for up to 24 h. After thawing, luciferase activity in 20 µl lystate was measured using a Bioorbiit 1253 luminometer (Bioorbit, Turku, Finland) and a commercial kit (Promega) following the manufacturer’s instructions. Vit-ERE-luciferase luminescence was then normalised according to the luminescence from the SV40-luciferase control reporter to provide a measure of ERE trans-activation. This vit-ERE-luciferase reporter has previously been shown (Pennie et al. 1998) to respond to both α and β forms of the oestrogen receptor (ERα and ERβ).

**PCR analysis of oestrogen receptors**

PCR was used to detect transcripts for ERα, ERβ and ERβ2 using normal rat pituitary tissue as an internal control. αT3-1 cells were grown to approximately 80% confluence in T75 flasks and rat pituitaries were collected from Alpk:AP rats (Animal Breeding Unit, AstraZeneca) and stored at −70 °C prior to RNA isolation. Total RNA was isolated using a guanidinium thiocyanate phenol-chloroform single-step extraction (Stratagene, Cambridge, Cambs, UK). First-strand cDNA was generated from total RNA using random hexamer primers, 4% of which were used for each PCR amplification. The following parameters were used for PCR amplification: 91 °C for 5 min, 54 °C for 5 min (45 °C for ERα amplification). After the addition of 2·5 units Taq DNA polymerase: 91 °C for 1 min, 54 °C for 1 min (45 °C for ERα amplification), 72 °C for 2 min, for 40 cycles with a final extension at 72 °C for 10 min.

The oligonucleotide primers 5'-TGCGGCTCCATGGAACACC-sense (+1176) and 5'-GCTGCAAGTCCAAGTTCCA-antisense (+1496) were used to amplify a 320 bp ERα product. The oligonucleotide primers 5'-GACAAGGACTGGTGCAATGAT-sense (+772) and 5'-AGGACATGTCAAGATTCCA-antisense (+1007) were used to amplify 235 and 289 bp ERβ and ERβ2 products respectively. The actin oligonucleotide
primers have been described previously (Grandien et al. 1995). All coordinates are relative to the ATG codon (A = +1).

After agarose gel electrophoresis in the presence of ethidium bromide, the PCR products were visualised by UV trans-illumination and photography. For confirmation of low-abundance expression the ERβ/β2 products were Southern blotted and hybridised to the antisense oligonucleotide, which had been radiolabelled using \([\gamma-32P]ATP\). The products were then visualised by autoradiography.

Flow cytometry and cell cycle analysis

Analyses were performed on a FACScalibur flow cytometer using CellQuest software (Becton Dickinson) for data acquisition. Cells were scraped from the culture vessels in PBS then centrifuged (300 g for 4 min at 21 °C), re-suspended in 2 ml PBS (pH 7-4), then re-centrifuged and suspended in 0·5 ml PBS. They were then fixed and permeabilised by adding 3 ml ethanol at −20 °C and stored at −20 °C for up to 4 weeks. Immediately before flow cytometry, the ethanol was removed by centrifugation (300 g for 4 min at 21 °C) and DNA was stained using 250 µl propidium iodide solution (0·15 mM propidium iodide, 0·0001% Triton X-100, 0·1 mM EDTA in PBS) for 1–6 h at room temperature in the dark. RNA was removed with 250 µl ribonuclease A (2 mg/ml in PBS). The stained cells were analysed at 100 000 cells/s and a minimum of 10 000 gated single cell events was acquired. The laser excitation wavelength was 488 nm and the photo-multiplier band pass filter for propidium iodide was 515 nm. DNA frequency histograms were generated from the Fl2 signals of appropriate single cell regions and cell cycle distribution was determined using Modfit LT Software (Verity Software House Inc., Topsham, ME, USA).

Quantification of GnRH receptors

The binding of \(^{125}\text{I}\)-labelled buserelin, a GnRH receptor agonist, to intact αT3-1 cells was used to determine the number and affinity of GnRH receptors as previously described (McArdle et al. 1992). Treated cells were washed in physiological saline solution (PSS: 127 mM NaCl, 1·8 mM CaCl\(_2\), 5 mM KCl, 2 mM MgCl\(_2\), 0·5 mM Na\(_2\)HPO\(_4\), 5 mM NaHCO\(_3\), 10 mM glucose, 0·1% BSA, 10 mM Hepes, pH 7·4), scraped from culture flasks, centrifuged (300 g, 5 min) and re-suspended in PSS containing 1 mg/ml bacitracin. Cells (8–16 × 10\(^6\)) were incubated for 30 min at room temperature with \(^{125}\text{I}\)-buserelin (0·236 nM) and a range of concentrations of non-radioactive buserelin (0·1 nM–1 µM). The incubations were then layered over 150 µl of a mixture of AR2000 silicon oil and bis(3,5,5-trimethylhexyl)phthalate (combined to a specific gravity of 1·01 g/ml) in Eppendorf microfuge tubes. After separating the cells from the incubation media by centrifugation through the inert oil mixture (2 min, 12 000 g), the microfuge tubes were frozen and the tips containing the cell pellets were cut off and the \(^{125}\text{I}\)-buserelin in the pellets was determined by \(\gamma\)-counting.

Measurement of Ins(1,4,5)P\(_3\) mass

\(^{1}
H\)Ins(1,4,5)P\(_3\) mass was measured using a previously characterised radioreceptor assay (Challiss et al. 1990). Cells incubated in 12-well plates were washed once and pre-incubated for 30 min in 1 ml Krebs’/Hepes buffer (10 mM Hepes, 4·2 mM NaHCO\(_3\), 10 mM glucose, 1·2 mM KH\(_2\)PO\(_4\), 4·7 mM KCl, 118 mM NaCl, 1·3 mM CaCl\(_2\), 1·2 mM MgSO\(_4\)) at 37 °C. Stimulation was with 150 µl GnRH in Krebs’/Hepes at the times and concentrations indicated. The reaction was terminated by adding 150 µl ice-cold 1 M trichloroacetic acid and the plates were left on ice for at least 15 min. Forty microlitres of 10 mM EDTA were added to a 160 µl aliquot from each well followed by 200 µl freshly prepared 1:1 (v/v) mixture of tri-n-octyl-amine and 1,2-trichloro-trifluoro-ethane. The samples were vortexed and incubated at room temperature for 15 min before centrifugation (10 000 g, 2 min, room temperature) followed by the removal of 100 µl of the upper aqueous phase of each tube, which were then added to 50 µl 25 mM NaHCO\(_3\) and stored at 4 °C for up to 3 days. Ins(1,4,5)P\(_3\) was quantified using 50 µl aliquots incubated for 30 min at 4 °C with 0·5 mg of bovine adrenal binding protein (McArdle et al. 1996, Willars et al. 1998) and 10 nCi \(^{3}
H\)Ins(1,4,5)P\(_3\) in a total volume of 200 µl. Bound and free ligand were separated by centrifugation at 38 000 g (5 min, 4 °C) followed by rapid removal of the supernatant by aspiration. \(^{3}
H\)Ins(1,4,5)P\(_3\) in the pellet was determined by \(\beta\)-counting (after dissolving in 100 µl 2 M NaOH) and read off a standard curve of 0·5 nM–10 µM non-radioactive Ins(1,4,5)P\(_3\). Ins(1,4,5)P\(_3\) values were normalised per mg protein, as determined with a microassay based on the Bradford method using a commercial kit (Sigma Chemical Co., Poole, Dorset, UK) and following the manufacturer’s instructions.

Measurement of total \(^{3}
H\)IP\(_x\) accumulation

Accumulation of \(^{3}
H\)IP\(_x\) in cells labelled by pre-incubation with \(^{3}
H\)inositol and stimulated in the presence of LiCl was used as a measure of PLC activity. Cells were cultured with the appropriate treatment(s) in 24-well plates in 1 ml media and 2·5 µCi \(^{3}
H\)myoinositol were added to each well for the final 12 h of incubation. After two PSS washes each well was stimulated for 15 min with 250 µl PSS containing 25 mM LiCl and the indicated concentration of GnRH or PACAP27. The stimulation was terminated by adding 1 ml water at 95 °C. The cells were lysed by freezing and thawing and

\[ ^{3}H \text{IP}_{x} \] was separated from free \[ ^{3}H \text{inositol} \] using anion exchange chromatography on Dowex-1 columns (formate form) as described (Huckle & Conn 1987). The total accumulated \[ ^{3}H \text{IP}_{x} \] was measured by \( \beta \)-counting and expressed as d.p.m. per \( \mu \text{g DNA} \), measured in identically treated cultures.

**Statistical analyses and data presentation**

The data shown are either from single experiments (typically the mean and s.e.m. of at least three replicate observations from a single experiment which is representative of at least three) or are pooled from multiple experiments (typically the mean and s.e.m. of at least three repeated experiments). Where data are pooled from multiple experiments, these are typically expressed as a percentage of an internal control as defined in the figure legends and all data given in the text are pooled from multiple experiments. Data comparisons were by means of Student’s t-test, accepting \( P>0.05 \) as statistically significant.

**Results**

We have previously shown that \( ^{3}H \text{thymidine} \) incorporation into \( \alpha \text{T3–1} \) cells is stimulated by oestradiol and inhibited by the competitive oestradiol receptor antagonist raloxifene (McArdle et al. 1992) and now extend these observations by demonstrating that the inhibitory effect of raloxifene can be overcome by oestradiol. As shown (Fig. 1A), oestradiol caused only a modest increase in \( ^{3}H \text{thymidine} \) incorporation (maximally 60%) in medium containing 2% charcoal–dextran-treated FCS. However, raloxifene clearly reduced \( ^{3}H \text{thymidine} \) incorporation and this effect was overcome by oestradiol, so that in the presence of \( 10^{-8} \text{M} \) or \( 10^{-6} \text{M} \) raloxifene, oestradiol had a much more dramatic effect (40-fold increase). With \( 10^{-8} \text{M} \) raloxifene, the EC\( _{50} \) for oestradiol action was approximately 5 nM, whereas with \( 10^{-6} \text{M} \) raloxifene this was increased by approximately 100-fold (calculated assuming identical efficacy). Similar results were obtained when a different antagonist (ICI 182,780) was used (not shown). The simplest interpretation of these observations is that, in spite of the use of medium containing only 2% (charcoal–treated) FCS, the medium contained sufficient oestrogenic activity to activate oestrogen receptors (Berthois et al. 1986), that this is blocked by raloxifene, and that the effect of this inhibitor can be competitively overcome by increasing concentrations of oestradiol. This interpretation was reinforced by experiments using an ERE-containing reporter (vit-ERE-luciferase) to measure oestrogen receptor activation. As with cell proliferation, oestrogen receptor activation (vit-ERE-luciferase normalised according to SV40-luciferase) was reduced by raloxifene and this inhibitory effect was overcome by increasing concentrations of oestradiol (Fig. 1B).

Recent work has revealed that oestrogen receptor subtypes selectively activate distinct EREs and that, in Cos-1 cells, the vitellogenin ERE can be activated by both ER\( _{a} \) and ER\( _{\beta} \), but is preferentially activated by ER\( _{\beta} \) (Pennie et al. 1998). We therefore used PCR to assess...
which oestrogen receptors are expressed in αT3–1 cells, using primers which would enable us to identify expression of ERβ2 as well as ERβ and including rat pituitary as a control tissue known to express ERα, ERβ and ERβ2 receptors. As expected, all three transcripts were detected in rat pituitary tissue (Fig. 2). In αT3–1 cells, ERα and ERβ transcripts were detected whereas ERβ2 was not (Fig. 2). Thus, the effects of oestradiol on vit-ERE-luciferase activity and on cell proliferation could reflect activation of ERα and/or ERβ.

The experiments above demonstrate that oestrogen receptor activation can be essential for αT3–1 cell proliferation and that the full extent of the oestrogen effect is most easily revealed by comparison of cells cultured with raloxifene with or without oestradiol. Flow cytometry was then used to define cell cycle distribution for αT3–1 cells cultured under these conditions using propidium iodide staining to define the proportion of cells having a 2 N (G0/G1), 4 N (G2/M) or 2–4 N (S-phase) complement of DNA. As shown (Fig. 3A), when cells were cultured in medium containing 10⁻⁸ M raloxifene, the vast majority were in the G0/G1 stage, few were in the G2/M stages and fewer still were in the S-phase (the phase of DNA synthesis). Oestradiol clearly increased the proportion of cells in the S-phase and G2/M-phases (Fig. 3A). Calculation of cell cycle distribution (using Modfit) revealed the competitive nature of the effects of raloxifene and oestradiol (Fig. 3B), and the close parallel to their effects on [³H]thymidine incorporation (Fig. 1). In cells cultured in normal medium (with 2% charcoal-dextran-treated FCS but without added oestradiol or raloxifene) approximately 12% of the cells were in the S-phase. Addition of 10⁻⁸ M raloxifene reduced the percentage of cells in S-phase to below 3%, while the addition of 10⁻⁸ M oestradiol completely overcame this inhibition by increasing S-phase levels to 15%. A similar increase in the level of S-phase cells was seen with the addition of 10⁻⁶ M oestradiol to cells cultured with 10⁻⁶ M raloxifene. These effects on the proportion of cells in the S-phase were paralleled by effects on G2/M-phase cells (not shown) and by changes in cell number. For example, cell number per well after 4-day treatment was increased from 40 000 ± 7000 in the presence of 10⁻⁸ M raloxifene up to 94 000 ± 1400 (n = 3) by the addition of 10⁻⁸ M oestradiol. This increase in cell number was due to stimulation of cell proliferation rather than inhibition of apoptosis because we were unable to detect cells with a less than diploid complement of DNA, after propidium iodide staining to determine the proportion of cells having a 2 N (G0/G1), 4 N (G2+M) or 2–4 N (S-phase) complement of DNA. As shown (Fig. 3A), when cells were cultured in medium containing 10⁻⁸ M raloxifene with or without 10⁻⁸ M oestradiol was inhibited by unlabelled buserelin. Curve fitting revealed a Kd value of 4.33 nM (log₁₀ 8.364 ± 0.17, data pooled from four experiments) in cells cultured with raloxifene alone, which did not differ significantly from that in cells cultured with raloxifene and oestradiol. Single-point binding assays (specific binding assessed by incubation with approximately 0.24 nM ¹²⁵I-buserelin) revealed that the binding to cells cultured with oestradiol and raloxifene was only 69 ± 11% (n = 4) of that in cells cultured with raloxifene alone (P > 0.01). Since oestradiol did not measurably alter the Kd value, single-point binding data are proportional to receptor number revealing that oestrogen receptor activation reduced GnRH receptor number by approximately 31%. A similar reduction in receptor

Figure 2 Detection of oestrogen receptor transcripts by PCR. RNA was extracted from rat pituitaries and αT3–1 cells and PCR was performed using primer pairs to amplify ERα (320 bp), ERβ or ERβ2 (235 and 289 bp respectively) and actin. After gel electrophoresis in the presence of ethidium bromide, the products were identified by UV trans-illumination and the identity of oestrogen receptor transcripts was confirmed by Southern blotting.

number was seen with oestradiol in the absence of raloxifene (McArdle et al. 1992). In spite of this reduction in GnRH receptor number, caused by oestradiol, the steroid increased the effects of GnRH on \( [\text{3H}]\text{IP}_x \) accumulation and Ins(1,4,5)P\(_3\) mass. As shown (Fig. 4B), GnRH caused a dose-dependent increase in \( [\text{3H}]\text{IP}_x \) in cells cultured in medium with raloxifene with or without oestradiol but the maximal response was greatly increased by oestradiol (e.g. the \( [\text{3H}]\text{IP}_x \) response to \( 10^{-6} \) M GnRH in cells cultured with raloxifene was only \( 52.4 \pm 17.8\% \) (\( n=3 \)) of that in cells cultured with raloxifene and oestradiol). Similarly, GnRH caused a dose-dependent increase in Ins(1,4,5)P\(_3\) mass (Fig. 5) and again the maximal response was increased by oestradiol (e.g. the \( [\text{3H}]\text{IP}_x \) response to \( 10^{-6} \) M GnRH in cells cultured with raloxifene was only \( 52.4 \pm 17.8\% \) (\( n=3 \)) of that in cells cultured with raloxifene and oestradiol). To test whether oestradiol influences the time-course of Ins(1,4,5)P\(_3\) elevation, cells cultured with raloxifene in the presence or absence of oestradiol were stimulated for between 5 and 300 s with \( 10^{-6} \) M GnRH. As shown (Fig. 5, inset) GnRH caused a rapid and sustained increase in Ins(1,4,5)P\(_3\) mass in both experimental groups. Although oestradiol clearly increased the magnitude of the response (e.g. response to \( 10^{-6} \) M GnRH at 10 s in cells cultured with raloxifene was only \( 36 \pm 3\% \) (\( n=3 \)) of that in cells cultured with raloxifene and oestradiol) it had no effect on the temporal profile of the response. Accordingly, the oestradiol-induced change in the maximal response (Fig. 5, main panel) cannot be attributed to alteration of temporal profile and consequent failure to detect the maximum response in cells cultured with raloxifene alone.

To test whether the effect of oestradiol on \( [\text{3H}]\text{IP}_x \) accumulation is specific to GnRH receptors, we determined the effects of PACAP27, which also activates PLC-coupled G-protein coupled receptors in \( \alpha T3-1 \) cells (Schomerus et al. 1993) after culture in medium with \( 10^{-7} \) M raloxifene and 0 or \( 10^{-7} \) M oestradiol. As shown (Fig. 6), PACAP27 caused a concentration-dependent increase in \( [\text{3H}]\text{IP}_x \) and, as with GnRH, the maximal response was greatly increased by oestradiol (e.g. the response to \( 10^{-6} \) M PACAP27 with raloxifene was only \( 19 \pm 4\% \) (\( n=3 \)) of that in cells cultured with raloxifene and oestradiol).

We next investigated whether oestradiol's effects on GnRH action are mediated by its effect on cell cycle distribution (or, more specifically, by its ability to increase the proportion of S-phase cells) reasoning that, if this were so, the effects of oestradiol would be mimicked by treatment with thymidine, which competitively inhibits ribonucleotide reductase activity, thereby depleting nucleotide pools of dCTP, inhibiting DNA synthesis and...
trapping cells in the S-phase. These experiments were carried out in media with 10% FCS and a reduced culture period (48 h) because in preliminary studies the low rate of cell division in 2% FCS medium necessitated long periods of exposure to thymidine (to achieve significant cell cycle coordination) and this was found to be cytotoxic. Using this modified protocol, 15 mM thymidine had a dramatic effect on the cell cycle distribution (Fig. 7A); in raloxifene-containing medium, thymidine increased the percentage of S-phase cells 2-3-fold from 11 to 25% and in medium with raloxifene and oestradiol, the proportion of cells in the S-phase was almost doubled from 16 to 31% by thymidine. These thymidine-induced increases in S-phase

Figure 4 Effects of oestradiol and raloxifene on GnRH receptor binding and GnRH-stimulated [3H]IP\(_x\) accumulation in αT3–1 cells. (A) Data from whole-cell binding assays using \(^{125}\)I-buserelin to determine the effect of oestradiol on GnRH receptor number and affinity. αT3–1 cells were pre-incubated for 4 days in media containing 2% charcoal-treated FCS with 10\(^{-8}\) M raloxifene and 10\(^{-8}\) M oestradiol (○) or with 10\(^{-8}\) M raloxifene alone (●) and then incubated for 30 min at room temperature with \(^{125}\)I-buserelin (0-236 nM) and the indicated concentrations of non-radioactive buserelin. Free and bound ligand were then separated by centrifugation through oil. The data are normalised per \(\mu\)g DNA and show means ± S.E.M. of four separate experiments. Curve fitting (Graphpad Prism, Graphpad Software Incorporation, San Diego, CA, USA) revealed a \(K_d\) of 4·33 nM for cells cultured with raloxifene alone and this was not significantly altered by addition of oestradiol. (B) Concentration–response relationship for stimulation of [3H]IP\(_x\) accumulation by GnRH after culture for 4 days in media containing 2% charcoal-treated FCS with 10\(^{-8}\) M raloxifene and 10\(^{-8}\) M oestradiol (○) or with 10\(^{-8}\) M raloxifene alone (●). The cells were labelled with [3H]inositol overnight and then stimulated with GnRH for 15 min (in the presence of LiCl) at the concentrations indicated. The data shown are the means ± S.E.M. (n = 3) from a single representative experiment, normalised per \(\mu\)g DNA.

Figure 5 Effects of oestradiol and raloxifene on the increase in Ins(1,4,5)P\(_3\) caused by GnRH in αT3–1 cells. Cells were pre-incubated for 4 days in media containing 2% charcoal-treated FCS with 10\(^{-8}\) M raloxifene and 10\(^{-8}\) M oestradiol (○) or with 10\(^{-8}\) M raloxifene alone (●) and then stimulated with GnRH for 2 min at the concentrations indicated. The means ± S.E.M. of three separate experiments normalised per mg protein are shown. The inset shows the time-course of the response to 10\(^{-7}\) M GnRH (means ± S.E.M. n = 3) from a single representative experiment.

Figure 6 Effects of oestradiol and raloxifene on PACAP27-stimulated [3H]IP\(_x\) accumulation. Cells were incubated for 2 days in media containing 10% FCS with 10\(^{-7}\) M raloxifene and 10\(^{-7}\) M oestradiol (○) or with 10\(^{-7}\) M raloxifene alone (●). Cells labelled with [3H]inositol overnight and stimulated with PACAP27 for 60 min (in the presence of LiCl) at the concentrations indicated. The data shown are means ± S.E.M. of three separate experiments, normalised per \(\mu\)g DNA.
coincided with 21–23% reductions in the percentage of G0/G1-phase cells (not shown) but were not paralleled by effects of thymidine on GnRH-stimulated Ins(1,4,5)P₃ production. As shown (Figs 6B and 7), the 5% increase in the proportion of cells in the S-phase caused by oestradiol was associated with a 2- to 3-fold increase in GnRH-stimulated Ins(1,4,5)P₃ generation. In contrast, the 15% increase in the proportion of cells in the S-phase caused by thymidine (irrespective of the presence or absence of oestradiol) was not associated with any increase in GnRH-stimulated Ins(1,4,5)P₃ generation.

The relationship between cell cycle distribution and GnRH receptor signalling was explored further by pharmacological manipulation of the cell cycle prior to the determination of its distribution, GnRH-stimulated [³H]IP₃ accumulation and GnRH receptor number (Table 1). As shown, hydroxyurea (a ribonucleotide reductase inhibitor) reduced the proportion of cells in the S-phase from 16 to 6%, whereas methotrexate (a folate reductase inhibitor) and thymidine increased this to 30 and 59% respectively. However, none of these treatments measurably altered GnRH receptor number or GnRH-stimulated Ins(1,4,5)P₃ production (Table 1).

**Discussion**

Gonadal steroids, including oestradiol, act centrally to influence GnRH secretion and, at the pituitary, to influence the responsiveness of gonadotrophs to GnRH. The mechanisms underlying such modulation of GnRH action are poorly understood although oestradiol can exert

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**Table 1** Correlation of cell cycle distribution with GnRH receptor binding and GnRH-stimulated [³H]IP₃ accumulation in aT3–1 cells. Cells were pre-treated for 2 days in media containing 10% FCS with one of the cell cycle synchronisation treatments listed. The proportion of cells in S-phase, GnRH-stimulated [³H]IP₃ accumulation (10⁻⁷ M GnRH for 15 min in the presence of LiCl) and GnRH receptor number (single-point assay using 0.236 nM ¹²⁵I-buserelin and 0 or 10⁻⁶ M unlabelled buserelin to define total and non-specific binding) were then each determined as described in the legends for Figs 3 and 4. The data shown are means ± S.E.M. (n = 3) from three separate experiments (each having triplicate observations).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells in S-phase (%)</th>
<th>Cells in G2/M-phase (%)</th>
<th>[³H]IP₃ accumulation (d.p.m./ng DNA)</th>
<th>¹²⁵I-buserelin binding (c.p.m./µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyurea</td>
<td>6.4 ± 1.6</td>
<td>5.1 ± 0.5</td>
<td>3.3 ± 0.8</td>
<td>18.5 ± 5.4</td>
</tr>
<tr>
<td>Colcemid</td>
<td>12.2 ± 3.2</td>
<td>67.8 ± 9.4</td>
<td>4.5 ± 1.0</td>
<td>10.5 ± 2.4</td>
</tr>
<tr>
<td>Control</td>
<td>15.9 ± 1.6</td>
<td>13.7 ± 2.5</td>
<td>6.1 ± 1.4</td>
<td>20.6 ± 5.1</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>27.3 ± 5.6</td>
<td>4.8 ± 4.4</td>
<td>3.7 ± 1.2</td>
<td>16.0 ± 3.6</td>
</tr>
<tr>
<td>Thymidine</td>
<td>54.1 ± 0.8</td>
<td>13.8 ± 0.7</td>
<td>5.8 ± 1.4</td>
<td>14.8 ± 5.8</td>
</tr>
</tbody>
</table>
coordinated effects on GnRH receptor number and on post-receptor signalling (Clayton et al. 1980, Mitchell et al. 1988, Drouva et al. 1990, Leong & Thorner 1991). In an earlier study we demonstrated that oestradiol causes a dose-dependent increase in [3H]thymidine incorporation and cell number in αT3–1 cells (McArdle et al. 1992). This proliferative effect was associated with a reduction in GnRH receptor number and an increase in maximal GnRH-stimulated [3H]IPx accumulation. This study not only established that oestradiol acts directly upon these gonadotroph-derived cells to influence GnRH receptor expression and receptor–effector coupling but also raised another intriguing possibility. Given the intimate and reciprocal relationships between G-protein coupled receptor signalling and cell cycle stage (Rostagno et al. 1996) and between steroid receptor signalling and cell cycle stage (Hu et al. 1994) we hypothesised: (i) that the proliferative effect of oestradiol is associated with alteration of the proportion of αT3–1 cells in different stages of the cell cycle; (ii) that cell cycle stage is a determinant of responsiveness to GnRH; and (iii) that the effects of oestradiol on GnRH receptor expression and signalling in these cells are a consequence of its effects on cell cycle distribution. We show here that (i) is correct, but have found no evidence in support of (ii) or (iii).

In our previous study, using phenol red-free medium supplemented with steroid-stripped serum we found that [3H]thymidine incorporation into αT3–1 cells was increased 3–to 5-fold by oestradiol and almost abolished by the competitive oestradiol receptor antagonist, raloxifene (McArdle et al. 1992). Since the concentration of oestradiol in the culture medium was estimated to be \(10^{-11}\) M, this could indicate either that these cells are extremely sensitive to low concentrations of oestradiol (e.g. that the low concentration of oestradiol remaining was sufficient to drive proliferation at 20–35% of the maximal rate) or that raloxifene exerts an oestrogen-receptor independent toxic effect on these cells. The data herein clearly support the first of these possibilities since: (i) the inhibitory effect of raloxifene on [3H]thymidine incorporation was reversed by oestradiol; (ii) the EC_{50} for stimulation of [3H]thymidine incorporation was increased at higher concentrations of raloxifene; and (iii) these effects on [3H]thymidine incorporation were mirrored by the effects of oestradiol and raloxifene on oestrogen receptor activity (as measured by vit-ERE-luciferase). Accordingly, it appears that oestradiol exerts a specific, dose-dependent and oestrogen-receptor mediated effect on [3H]thymidine incorporation into αT3–1 cells which can be competitively inhibited by raloxifene. Although the vit-ERE-luciferase reporter is preferentially activated in Cos-1 cells by ERβ (Pennie et al. 1998), it is also activated by ERα and αT3–1 cells apparently express both ERα and ERβ. Accordingly, although the culture conditions used (e.g. raloxifene in the presence and absence of oestradiol) provide a simple means of achieving minimal and maximal ER activation for comparative purposes, we were unable to define the relative contribution of the ER subtypes to the observed effects of oestrogen.

Using flow cytometry to define cell cycle distribution, we have found that the stimulatory effects of oestradiol on [3H]thymidine incorporation (and their competitive inhibition by raloxifene) are paralleled by increases in the proportion of the cells in the S- and G2/M-phases of the cell cycle, at the expense of the proportion in the G1/G0 stages. When the cell doubling time is known, the proportional distribution of cells in different cell cycle phases can be directly related to the duration of those phases but we have found that detachment of cells from plates prevents accurate assessment of the doubling time of αT3–1 cells by simple daily cell counts. Accordingly, we have performed parallel studies (not shown) using a stable fluorescent marker, PKH2, which distributes into cell membranes (Horan et al. 1990). By monitoring mean fluorescence per cell by flow cytometry and the rate of reduction in this parameter as the cells divide, we have estimated that the doubling time of αT3–1 cells cultured in medium with raloxifene is approximately 73 h and that this is reduced to approximately 39 h by addition of oestradiol (not shown). Using these figures to estimate the duration of the cell cycle stages reveals that the oestradiol-induced increases in the proportion of cells in the S- and G2/M-phases are not due to changes in the duration of these phases but, instead, reflect a reduction (from approximately 68 to 26 h in media containing 2% FCS) in the mean duration of the G1/G0 stages. Accordingly, the mitogenic effect of oestradiol on αT3–1 cells reflects either recruitment of quiescent cells into the G1-phase or driving the cells through G1 and/or the G1/S transition. These observations are compatible with oestradiol effects in other cell types, such as MCF-7 cells, in which oestradiol increases the proportion of cells in the S-phase and oestrogen receptor antagonists block entry into the S-phase (Prall et al. 1997). It is important to note, however, that αT3–1 cells were generated by targeted tumorigenesis with the SV40 T antigen (Windle et al. 1990). In many systems SV40 T causes neoplasia (or increases the probability of its occurrence) by binding to growth suppresser proteins such as p53 and pRB (the product of the retinoblastoma susceptibility gene). pRB is thought to block exit from G1 and this block can be removed either by phosphorylation or by complexing with the SV40 T antigen. It remains to be seen whether oestradiol is a sufficient stimulus for proliferation of gonadotrophs in which this block has not been removed by SV40 T (e.g. non-immortalised foetal or neonatal gonadotrophs).

Interestingly, the dose–response relationship for stimulation of the luciferase reporter by oestradiol was right shifted when compared with the effects on [3H]thymidine incorporation and proportion of cells in the S-phase so that, in the presence of \(10^{-8}\) M raloxifene, \(10^{-8}\) M

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Oestradiol had maximal effects on proliferation and cell cycle distribution but did not maximally activate reporter expression. Thus, the potency of oestradiol at stimulation of proliferation in these cells is partly due to the fact that maximal stimulation of this response occurs with only a proportion of oestrogen receptors activated (e.g. receptor ‘reserve’ or ‘spare’ receptors exist for this response). The difference in extent of receptor reserve for stimulation of proliferation and reporter expression could reflect the increased efficiency of oestrogen receptor signalling within the context of normal DNA, with additional transcription factors and co-activators present, and the fact that this is not mirrored in transcriptional regulation of the reporter.

The proliferative effect of oestradiol was paralleled by its effect on GnRH receptor number (reduction to approximately 69% of that with raloxifene alone) and an increase in GnRH-stimulated [3H]IP3 accumulation (to approximately double that of raloxifene alone). Similarly, the maximal effect of GnRH on Ins(1,4,5)P3 mass was increased by oestradiol (to approximately double that with raloxifene alone) and this occurred without any alteration in the temporal profile of the response. Oestradiol appears, therefore, to increase these responses by increasing GnRH-stimulated PLC activity, rather than by increasing specific activity of the phosphatidylinositol 4,5 bis-phosphate pool (a possible alternative interpretation of the [3H]IP3 dose–response data) or by altering the kinetics of the Ins(1,4,5)P3 response (a possible alternative interpretation of the Ins(1,4,5)P3 dose–response data). This increase in GnRH-stimulated PLC activity occurs in spite of a reduction in GnRH receptor number indicating that the ratio of maximal inositol phosphate response to receptor number is increased by oestradiol. To test whether this increase in receptor–effector coupling efficiency is specific for the GnRH receptor we determined effects of oestradiol on PLC activation by PACAP27. This polypeptide acts via PLC and adenyl cyclase-coupled PAC1 receptors in these cells and stimulates [3H]IP3 accumulation, but with a much lower maximal response (5–20%) than that to GnRH (Schomerus et al. 1993). As expected, PACAP27 caused a dose-dependent increase in [3H]IP3 accumulation. The maximal response to PACAP27 was markedly increased (5- to 6-fold) by oestradiol (as compared with raloxifene alone) demonstrating that such amplification is not limited to the GnRH receptor and most likely, therefore, reflects alterations in amount, activity or compartmentalisation of downstream signalling proteins. In this regard, the most obvious candidates are the subunits of Gq/11. It has been shown that oestradiol exposure can influence G-protein subunit expression in rat anterior pituitaries in vivo (Bouvier et al. 1991) but it is not yet clear whether any such effect is exerted directly upon gonadotrophs or αT3–1 cells. An alternative possibility is that oestradiol simply increased PACAP receptor number in αT3–1 cells, but this is unlikely because it had much less pronounced effects (2-fold) on PACAP27-stimulated cAMP accumulation (not shown).

Having demonstrated effects of oestradiol on cell cycle distribution and GnRH signalling in αT3–1 cells, we sought to establish whether they are causally related. We initially attempted to define effects of oestradiol in cells rendered quiescent by culture to confluence. However, this was unsuccessful because even after extended culture, the proportion of S-phase cells was not appreciably reduced. Instead, many cells detached from the culture vessels as they approached confluence, leaving a surface for continued cell division. As an alternative method we used thymidine to achieve an S-phase block, reasoning that if the effects of oestradiol on GnRH signalling were due to its increase in S-phase cells, the signalling effects would be both mimicked and blocked by thymidine. However, this was not the case. Thymidine caused a pronounced increase in the proportion of cells in the S-phase (with and without oestradiol) but did not mimic the effect of oestradiol on GnRH signalling. Indeed, manipulations of cell cycle distribution with thymidine, methotrexate, colcemid and hydroxyurea all failed to appreciably alter GnRH-stimulated [3H]IP3 accumulation or GnRH receptor expression, and overall, experiments with these manipulations revealed no significant correlation between the proportion of cells in the S-phase or G2/M-phase and the response of these cells to GnRH.

In summary, we confirm that oestradiol has a trophic effect on αT3–1 cells and show that, when raloxifene is used to competitively block effects of endogenous oestrogenic compounds in the culture medium, the proliferation of these cells is almost entirely oestradiol dependent. This mitogenic effect of oestradiol could be mediated by ERα and/or ERβ, both of which are expressed by these cells and the steroid appears to drive the cells through the G0/G1-phase into the S-phase, thereby reducing the doubling time and increasing cell number, [3H]thymidine incorporation and the proportion of cells in the S-phase and G2/M-phases. These trophic effects are paralleled by a modest reduction in GnRH receptor number and a pronounced increase in the efficiency with which these receptors stimulate PLC. The mechanisms underlying the latter effect are unknown, but it apparently reflects alterations downstream of the GnRH receptor (because effects of PACAP27 are similarly amplified by oestradiol) and is not due to oestradiol’s effects on cell cycle distribution (because a range of manipulations revealed no correlation between GnRH signalling and cell cycle distribution).

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