Direct effects of prolactin and dopamine on the gonadotroph response to GnRH

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Abstract

The intrapituitary mechanisms underlying the inhibitory actions of hyperprolactinaemia on the reproductive axis remain unclear. Previous work on primary pituitary cultures revealed combined suppressive effects of prolactin (PRL) and dopamine on the gonadotrophin response to GnRH. However, whether these effects occur directly at the level of the gonadotroph and are accompanied by changes in gene expression is still unresolved. Here, αT3-1 and LBT2 cells were used to investigate the effects of PRL and dopamine on gonadotrophin synthesis and release in gonadotroph monocultures under basal and GnRH-stimulated conditions. PRL receptor and dopamine receptor mRNA expressions were first determined by RT-PCR in both cell lines. Then, PRL and the dopamine agonist bromocriptine (Br), alone or in combination, were shown to block the maximal α-subunit and LHβ-subunit mRNA responses to a dose-range of GnRH. The LH secretory response was differentially affected by treatments. GnRH dose-dependently stimulated LH release, with a 4–5 fold increase at 10−8 M GnRH. Unexpectedly, PRL or Br stimulated basal LH release, with PRL, but not Br, enhancing the LH secretory response to GnRH. This effect was, however, completely blocked by Br. These results reveal direct effects of PRL and dopamine at the level of the gonadotroph cell, and interactions between these two hormones in the regulation of gonadotrophin secretion. Moreover, uncoupling between LH synthesis and release in both the basal and the GnRH-stimulated responses to PRL and dopamine was clearly apparent.


Introduction

Hyperprolactinaemia is a major cause of amenorrhoea in humans and has been shown to be associated with the suppression of gonadotrophin secretion and impaired fertility in this (Delvoye et al. 1978, Kremer et al. 1991, Blackwell 1992) and other (Bartke et al. 1977, McNeilly et al. 1978) mammalian species. While the specific mechanisms underlying prolactin (PRL) effects on gonadotrophin release are still unresolved, it has become increasingly apparent that, in addition to the established actions of PRL at the level of the hypothalamus affecting gonadotrophin-releasing hormone (GnRH) output, interactions between the gonadotrophic and PRL axes occur at the level of the pituitary gland. Indeed, close associations between gonadotrophs and lactotrophs were originally identified in the rodent pituitary, where cell-to-cell communication in the form of adherans junctions (Horvath et al. 1977) and specific gap junction coupling (Morand et al. 1996) could be established. These morphological associations between the gonadotroph and lactotroph cells have also been identified in the pituitaries of larger vertebrates, where isolated gonadotrophs were shown to be situated within clusters of lactotrophs (Tortonese et al. 1998, Gregory et al. 2000).

Functional observations further support the existence of intrapituitary effects of PRL on gonadotrophin secretion. PRL receptor mRNA and protein have been detected within the pituitary gland of the rat (Chiu et al. 1992), and treatment of cultured pituitary fragments with PRL resulted in the suppression of both basal and GnRH-stimulated luteinizing hormone (LH) release (Cheung 1983). In humans, the pituitary LH response to GnRH is reduced during lactational amenorrhoea when PRL concentrations are elevated (Jeppsson et al. 1974, Le Maire et al. 1974, Andreassen & Tyson 1976), and this has been associated with LH pulses of low amplitude (Tyson et al. 1978, Glasier et al. 1984). Similarly, in subhuman primates, exogenous PRL was shown to suppress the LH response to GnRH (Maneckjee et al. 1976). An additional evidence for a direct pituitary action of PRL on the gonadotrophic axis was revealed by a study reporting the selective expression of PRL receptors in gonadotroph cells of the pars distalis and pars tuberalis of the ovine pituitary, a species that shows overt temporal changes in fertility throughout the year in response to photoperiod (Tortonese et al. 1998). More recently, our laboratory demonstrated a seasonal modulation of the combined suppressive effects of PRL and dopamine on the gonadotrophi response to GnRH (Gregory et al. 2004).

An immortalised gonadotroph cell line, αT3-1, has been shown to express D2-type dopamine receptors and it has been suggested that these receptors negatively regulate pituitary α-subunit gene expression in association with a
cAMP-dependent pathway (Kanasaki et al. 2002), making it possible to investigate the direct effect of dopamine on a gonadotroph monoluculture. αT3-1 is a mouse-derived cell line which expresses the mouse gonadotrophin α-subunit gene and synthesises and secretes the α-subunit protein (Windle et al. 1990). These cells also express GnRH receptors within the concentration range observed in primary gonadotrophs, and activation of these receptors evoked an equivalent response to GnRH by increasing the α-subunit mRNA in a dose-dependent manner (Windle et al. 1990, Horn et al. 1991). As the αT3-1 cell line was immortalised at a relatively early stage of development, it does not produce the gonadotrophin β-subunits. A similar cell line, LBT2, was created using tumorigenesis in transgenic mice carrying the LHβ-subunit regulatory region (Alarid et al. 1996). These cells express the gonadotrophin α-subunit, the LHβ-subunit, GnRH receptor mRNA, and respond to GnRH with dose-dependent increases in LH secretion, exhibiting functional characteristics consistent with those of normal pituitary gonadotrophs. Further studies have demonstrated LH secretion via a regulated pathway, and changes in GnRH receptor expression in response to signalling by GnRH and steroid hormones (Turgeon et al. 1996). As the LBT2 cells represent αT3-1 cells arrested at a later stage of development, it seems probable that the LBT2 cell line will also express D2 dopamine receptors. The expression of PRL receptors by either of these cell lines is yet to be determined. If present, the αT3-1 and/or LBT2 cell lines could offer a useful tool with which to investigate further the direct effects of PRL and dopamine on gonadotrophs.

The aim of this study was first to determine the expression of PRL receptors by αT3-1 and LBT2 cell lines, as well as the expression of dopamine receptors by LBT2 cells. Subsequently, these cell lines were used to investigate the combined suppressive effects of PRL and dopamine by measuring the gonadotrophin α-subunit and LHβ-subunit mRNA responses to PRL and a dopamine agonist, alone or in combination, while simultaneously assessing the LH secretory response in the presence or absence of GnRH.

Materials and Methods

Cell culture and experimental design
αT3-1 and LBT2 gonadotroph cells (a gift from Dr Pamela Mellon, University of California, San Diego, CA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% fetal calf serum (PAA Laboratories, Linz, Austria) and 1% penicillin–streptomycin (Gibco), and maintained at 37 °C in an atmosphere of 95% air and 5% CO2. The cells were plated onto six-well plates at 3 × 10⁵ cells/well for 4 days, and then either treated for 24 h or washed in PBS (calcium-, magnesium- and sodium bicarbonate-free, Gibco) and incubated in serum-free DMEM overnight before treatment was applied for 90 min. The cells were treated with:

- i) buffer (Control), ii) GnRH (10⁻⁷ M), iii) bromocriptine (Br) (10⁻⁸ M), iv) PRL (500 ng/ml), v) GnRH + Br, vi) GnRH + PRL, and vii) GnRH + Br + PRL. The optimal GnRH dose required to generate a maximal mRNA response (i.e. 10⁻⁷ M) was previously determined by dose–response tests at 90 min and 24 h. The doses of Br (a specific dopamine-D2 receptor agonist) and PRL employed in this study were selected on the basis of their previously observed effectiveness at inducing a biological response in preliminary studies, and in previous work in αT3-1 (Kanasaki et al. 2002) and primary (Gregory et al. 2004) cultures. Following incubation, the medium was aspirated off prior to cell lysis and RNA extraction. GnRH and bromocriptine were purchased from Sigma–Aldridge Ltd, whereas PRL was obtained from Dr A F Parlow (ovine PRL, lot AFP-10692c, National Institute of Diabetes and Digestive and Kidney Diseases, NIDDKD, Torrance, CA, USA).

RT-PCR
Total RNA was extracted from αT3-1 and LBT2 cells, homogenised using Qiashredder columns (Qiagen) and purified using an RNeasy mini kit (Qiagen). mRNA was reverse-transcribed into single-stranded cDNA using oligo(dT)18 primer reverse transcriptase (Roche Diagnostics). cDNA samples were then subjected to PCR amplification using primers specific to: i) GAPDH, ii) a 645 bp product common to the short and long forms of the PRL receptor, iii) the long form of the D2 dopamine receptor, iv) the gonadotrophin α-subunit and v) the LHβ-subunit (for sequences see Table 1). PCR amplification was carried out using a MJ research PTC-200 Peltier Thermal Cycler.

The conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by an amplification step at temperatures appropriate for each primer set (see Table 1) for 45 s, and extension at 72 °C for 1 min. The optimal number of amplification cycles for quantification of the products of each primer set had been previously determined by titration experiments. These were 23 cycles for the α-subunit and 25 cycles for the LHβ-subunit expression. For the assessment of the expression of PRL and dopamine receptors, 35 cycles were used. Following amplification, the final 10 min extension step was carried out at 72 °C. PCR products were separated by electrophoresis on a 1.5% agarose gel containing 0-1 μg/ml ethidium bromide and quantified by scanning densitometry using Bio-Rad Quantity One Quantification Software. The amounts of PCR products of each subunit were normalised to those of the PCR products of GAPDH in each sample and the values were converted to a percentage of the control in order to allow a direct comparison between individual experiments (Kanasaki et al. 2002).

RIA
The concentration of LH in culture medium following treatment was measured by RIA using reagents supplied by NIDDKD’s National Hormone and Peptide Program and A F
Table 1  Annealing temperatures and primer sequences used for PCR amplification of prolactin (PRL) receptor, D2 dopamine receptor and gonadotrophin α- and luteinizing hormone β (LHβ)-subunit genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Annealing temperature (°C)</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>PRL-R</td>
<td>60</td>
<td>5′: GCA GAT GGA GGA CTT CCT ACC AAT TA</td>
</tr>
<tr>
<td>D2 Dopamine-R</td>
<td>52</td>
<td>3′: GCA GGT CAC CAT GCT ATA GCC CTT</td>
</tr>
<tr>
<td>α-Subunit</td>
<td>55</td>
<td>5′: AGA GCC AAC CTG AAG ACA CCA</td>
</tr>
<tr>
<td>LHβ-Subunit</td>
<td>57</td>
<td>3′: GAT GAT GAA CAC ACC GAG AAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′: ACT TTA TTA TCT AGG GGT TGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3′: TAT AAG GGA TGT AAC CTT AAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′: TGG CTG CTG CTG AGC CCA AGT G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3′: CAG GTC ATT GGT TGA GTC CTG G</td>
</tr>
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Parlow, with all samples being assayed in duplicate in the same assay. The reference preparation employed was mouse LH (AFP-5306A), with rat LH (AFP-11536B) for iodination, and rat LH antiserum (NIDDK-anti-rLH-S-11) as the primary antibody. The minimum detectable concentration was 0.1 ng/ml and the intra-assay coefficient of variation was 7.2%.

Statistical analysis

In both the αT3-1 and LβT2 cultures, six doses of GnRH were employed for dose–response tests, while for treatment effects, seven experimental groups were used. For each group, four wells were assigned to each treatment, and the experiments were carried out three times with reproducible results. The values are expressed as means ±S.E.M. The effects of treatments on the gonadotrophin subunit mRNA and secretory responses were examined by ANOVA, followed by pairwise comparison using Fisher’s test to determine specific differences among treatments. Treatment effects were considered statistically significant at P<0.05.

Results

Expression of PRL and dopamine D2 receptors in αT3-1 cells

In the first series of studies, total RNA from αT3-1 cells was analysed by RT-PCR to assess the presence of PRL receptors and confirm the expression of dopamine D2 receptors. As shown in Fig. 1, both the PRL receptor (645 bp) and the long form of the dopamine D2 receptor (458 bp) mRNA were detected in αT3-1 cells.

Effects of PRL and dopamine on GnRH-induced α-subunit gene expression in αT3-1 cells

Dose–response tests confirmed the ability of αT3-1 gonadotroph cells to respond to GnRH under culture conditions. Acute (90 min) treatment resulted in a clear dose-dependent response, with a 2.5-fold increase in α-subunit mRNA at 10⁻⁷ M GnRH (P<0.01; Fig. 2A). In contrast, chronic GnRH treatment (24 h) showed a moderate increase at all
concentrations tested ($P < 0.05$; Fig. 2B). In the subsequent phase of this second series of studies, the acute application of PRL or Br did not modify basal $\alpha$-subunit mRNA expression (Fig. 3). However, the $\alpha$-subunit response to GnRH was totally suppressed when PRL or the dopamine agonist were applied alone or in combination ($P < 0.01$). Similarly, in chronic (24 h) studies, a sub-maximal $\alpha$-subunit response to GnRH was observed after treatment with PRL or Br (data not shown).

Expression of PRL and dopamine D$_2$ receptors in L$\beta$T2 cells

In the third series of studies, total RNA from L$\beta$T2 gonadotroph cells was analysed by RT-PCR to examine the expression of PRL and D2 dopamine receptors by this cell line. As shown in Fig. 1, both PRL receptor (645 bp) and the long form of the dopamine D$_2$ receptor (458 bp) mRNA were detected in L$\beta$T2 cells.

Effects of PRL and dopamine on GnRH-induced LH$\beta$-subunit gene expression in L$\beta$T2 cells

In the fourth series of studies, L$\beta$T2 cultures were treated acutely (90 min) with increasing doses of GnRH ($10^{-9} - 10^{-6}$ M). A clear dose–response was observed, with maximal stimulation at $10^{-7}$ M GnRH ($P < 0.01$) (Fig. 4A). In the second phase of this series of studies, treatment with PRL or Br, alone or in combination, completely suppressed the LH$\beta$-subunit mRNA response to GnRH ($P < 0.01$) (Fig. 5A), whereas the same treatments had no effect on basal mRNA expression.

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**Figure 2** Effects of (A) acute (90 min) or (B) chronic (24 h) treatment of $\alpha$T3-1 cells with increasing doses of GnRH ($10^{-9} - 10^{-6}$ M) on gonadotrophin $\alpha$-subunit mRNA expression. Values expressed as mean percentage of Control ± S.E.M. ($^*P < 0.05$, $^{**}P < 0.01$, relative to Control).

**Figure 3** Gonadotrophin $\alpha$-subunit mRNA response in $\alpha$T3-1 cultures following acute (90 min) treatment with: (i) buffer (Control), (ii) GnRH (10$^{-7}$ M), (iii) bromocriptine (Br) (10$^{-8}$ M), (iv) PRL (500 ng/ml), (v) GnRH + Br, (vi) GnRH + PRL and (vii) GnRH + Br + PRL. Values are expressed as a mean percentage of the Control ± S.E.M. (NS, non-significant, $^{**}P < 0.01$).

**Figure 4** Effects of acute (90 min) treatment of L$\beta$T2 cell cultures with increasing doses of GnRH ($10^{-9} - 10^{-6}$ M) on (A) expression of LH$\beta$-subunit mRNA (values expressed as mean percentage of Control ± S.E.M.) and (B) LH secretory response (as measured by RIA) ($^{**}P < 0.01$, relative to Control).
The results of this study demonstrate the expression of functional PRL and dopamine receptors in gonadotroph cells. Indeed, suppression of the gonadotrophin mRNA response to GnRH at the level of both the α- and β-subunits by PRL and a dopamine agonist (Br) was clearly apparent. Contrary to these observations, measurements of the LH secretory response in LβT2 gonadotrophs revealed that single applications of PRL or Br were capable of stimulating basal LH secretion under these culture conditions. Moreover, the LH response to GnRH was enhanced by PRL, while the co-application of Br with GnRH did not alter the LH secretory response in comparison with the stimulation by GnRH alone. Interestingly, when PRL was combined with the dopamine agonist, the PRL-induced increase in the LH secretory response to GnRH was completely abolished.

While PRL is known to participate in the central control of fertility with well-documented effects in the hypothalamus, it is becoming increasingly apparent that PRL can also act directly at the level of the pituitary gland to regulate gonadotrophin output. Early morphological studies reporting intercellular interactions between gonadotroph and lactotroph cells in the rodent pituitary (Horvath et al. 1977) and the subsequent functional observations revealing hyperprolactinaemia-induced reduction of the LH secretory response to GnRH in rodents and humans (Cheung 1983) were subsequently supported by the identification of the two forms of the PRL receptor (gene and protein) within this tissue (Ouhit et al. 1993), thus confirming that the infrastructure required for a direct pituitary action of PRL is indeed present. In the current study, the demonstration that both the PRL receptor and the DA receptor are expressed within the gonadotroph has important clinical implications, since it reveals that the gonadotroph itself can be a point for the control of fertility in hyperprolactinaemia-induced amenorrhoea. Indeed, the suppression of both gonadotrophin subunits mRNA responses to GnRH by PRL and bromocriptine shows that the secretory effects of this treatment originally detected in primary cultures are likely to be accompanied by changes in gene expression.

The inhibition of the mRNA response to GnRH by PRL and Br (alone or in combination) may involve a cross-talk between the intracellular signalling pathways activated by GnRH and those activated by PRL and/or dopamine. The common components in the signalling of one or more of these hormones are likely to be mediating these interactions. GnRH receptors are members of the G-protein-coupled receptor family; ligand binding leads to the activation of Gq and/or G11 proteins (Shah & Milligan 1994), stimulation of phospholipase C (PLC), production of diacylglycerol (DAG) and activation of various protein kinase C (PKC) isoforms (Harris et al. 1997). Downstream of this pathway, GnRH activates the extracellularly regulated kinase (ERK) cascade of the mitogen-activated protein kinase (MAPK) signalling pathway via a PKC and tyrosine kinase-dependent mechanism (Naor et al. 2000). Simultaneously, GnRH induces intracellular calcium mobilisation and extracellular calcium influx (Stojilkovic et al. 1994). PRL receptors are members of the cytokine family (Kelly et al. 1993) and are coupled to Janus Kinase 2, a tyrosine kinase. The activation of these receptors leads to phosphorylation, and thus activation of specific transcription factors from the signal transducer and activator of transcription (Stat) protein family (Ihle 1996). The PRL receptor has also been implicated in the stimulation of MAPK cascades (Piccoletti et al. 1997), and connections...
between the JAK-Stat and MAPK pathways have been suggested (Bole-Feyso et al. 1998). Dopamine receptors, like GnRH receptors, are G-protein-coupled; these receptors are linked to G_{i/o} and/or G_{11} proteins (Enjalbert et al. 1988). The activation of the dopamine receptor results in an immediate increase in intracellular potassium and a consequent reduction in the intracellular free calcium, leading to an inhibition of release from secretory granules (Gregerson 2001). Additionally, dopamine acts to suppress PLC, thus reducing calcium release from the endoplasmic reticulum (Caccavelli et al. 1992). The principal pathway responsible for reduced gene transcription following activation of the dopamine receptor results in the inhibition of adenyl cyclase (Tausig & Gilman 1992). The principal pathway responsible for reduced gene transcription following activation of the dopamine receptor results in the inhibition of adenyl cyclase (Tausig & Gilman 1992). The activation of the ERK/MAPK pathway, the calcium mobilisation associated with gonadotrophin release (Zhu et al. 2002), it is likely that PRL-induced activation of PKC accounts for the observed enhancement the LH secretory response to GnRH. Overall, the present findings are consistent with those of an early study in post-partum women where the LH response to exogenous GnRH was suppressed during the first month after parturition but enhanced above follicular phase levels in the second month (Keye & Jaffe 1976). In the case of dopamine, the blockade of the PRL-induced enhancement of the secretory response to GnRH by the co-application of bromocriptine is an important observation, which corroborates previous findings in primary cultures (Gregory et al. 2004). If PRL is indeed inducing the observed enhancement through up-regulation of certain signalling components, dopamine is likely to be blocking these effects.

It thus becomes apparent that the paradoxical effects of PRL (i.e. whether stimulatory or inhibitory) on LH synthesis and release are likely to be dependent on the dopaminergic tone within the tissue. This may explain the apparent discrepancies in the reported associations between PRL and gonadotrophin secretion during lactational amenorrhoea in humans. Specifically, whereas some studies have provided evidence for a negative correlation between gonadotrophin secretion and PRL at this time (Jeppsson et al. 1974, Le Maire et al. 1974, Andreassen & Tyson 1976, Delvoye et al. 1978, Duchen & McNeilly 1980) and that the duration of puerperal amenorrhoea is positively associated with the level of prolactinaemia (Diaz et al. 1989, 1991), others have found no correlation between the two (Nunley et al. 1991, Tay et al. 1992). In a study where dopamine and opioid antagonists were administered to breastfeeding women, the resulting increases in endogenous PRL secretion did not significantly affect the gonadotrophin responses to an injection of GnRH (Tay et al. 1993). Since in the present study the LH secretory response to the secretagogue was enhanced by PRL, and that effect was unambiguously blocked by DA, it would be thus pertinent to consider what mechanisms could develop in vivo in some circumstances to counteract the suppressive effects of hyperprolactinaemia on the reproductive axis reported by others and/or the robust treatment effects observed here in vitro.

In conclusion, this study has demonstrated the expression of functional PRL and dopamine receptors by gonadotroph cells. These receptors were shown to mediate the suppressive effects of PRL and a dopamine agonist on the gonadotrophin mRNA response to GnRH, not only at the level of the common ß-subunit but also affecting the LHß-subunit. In contrast, the LH secretory response to GnRH was augmented by PRL. While the dopamine agonist did not share this ability, it was able to block the enhancing effect of PRL when the two were co-applied. These results have important clinical implications since they provide evidence for uncoupling between gonadotrophin synthesis and release in response to treatments; they also support previous observations in primary cultures revealing that the combined suppressive effects of PRL and dopamine on the gonadotrophin response to GnRH are accompanied by alterations in gonadotrophin subunit mRNA expression.

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