Dopaminergic regulation of avian prolactin gene transcription

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Abstract

It is well documented that prolactin (PRL) release and PRL gene expression in birds are controlled by the tonic stimulation of hypothalamic vasoactive intestinal peptide (VIP). However, there is good evidence that dopamine (DA) exerts both stimulatory (at the hypothalamic level) and inhibitory (at the pituitary level) effects on PRL secretion. The interactions between VIP and DA in the regulation of PRL gene transcription are not known. This study was designed to examine the effects of a D_2 DA receptor agonist (D_2AG; R(−)-propylnorapomorphine HCl) on basal and VIP-stimulated PRL gene transcription rate, PRL mRNA steady-state levels, PRL mRNA stability and PRL release from cultured turkey anterior pituitary cells. The D_2AG (10^{-10} M) completely inhibited the stimulatory effect of VIP (10^{-7} M) upon nascent PRL mRNA as determined utilizing a nuclear run-on transcription assay. To examine further the effect of the D_2AG on PRL mRNA post-transcriptional events, anterior pituitary cells were treated with different concentrations of D_2AG (10^{-12}–10^{-4} M). Semi-quantitative RT-PCR and RIA were performed to determine the levels of PRL mRNA and PRL content in the medium respectively. The results show that D_2AG inhibited VIP-stimulated PRL mRNA steady-state levels as well as basal and VIP-stimulated PRL release, effects which were diminished by the D_2 DA receptor antagonist, S(−)-eticlopride HCl (10^{-10} M). Actinomycin D (5 μg/ml), an inhibitor of mRNA synthesis, was used to assess the effect of D_2AG on PRL mRNA stability in response to VIP. The stimulatory effect of VIP on PRL mRNA stability was completely negated by the D_2AG (from a half-life of 53·0±2·3 h in VIP-treated cells to 25·5±1·6 h in D_2AG+VIP-treated cells, $P \leq 0{\cdot}05$). These results support the hypothesis that VIP and DA play a major role in the regulation of PRL gene expression in avian species, at both the transcriptional and post-transcriptional levels. In addition, these findings suggest that the DAergic system inhibits PRL release and synthesis by antagonizing VIP at the pituitary level via D_2 DA receptors.

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Introduction

It has been established for some time that prolactin (PRL) secretion in avian species is under tonic stimulatory control exerted by the hypothalamus (Kragt & Meites 1965, Bern & Nicoll 1968) and several lines of evidence indicate that vasoactive intestinal peptide (VIP) is the PRL-releasing factor (PRF) in birds (for review see El Halawani *et al.* 1997). In contrast to birds, the identity of the physiological PRF in mammals remains unascertained (Ben-Jonathan *et al.* 1989, Freeman *et al.* 2000, Taylor & Samson 2001). A group of factors, mostly neuropeptides, have been shown to exhibit stimulatory effects on PRL release. Among them are thyrotropin-releasing hormone (TRH) (Curlewis *et al.* 2002, Kanasaki *et al.* 2002, Yuan & Pan 2002) and the newly described PRL-releasing peptides (Hinuma *et al.* 1998, Curlewis *et al.* 2002). However, PRL release from the anterior pituitary is regulated principally by inhibitory influences imparted by the tuberoinfundibular dopamine (DA) system (Ben-Jonathan *et al.* 1977, Pasqualini *et al.* 1988, Ben-Jonathan & Hnasko 2001). In avian species, the role of DA in regulating PRL secretion is still largely obscure. DA, or the DA agonist,

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apomorphine, reduces PRL secretion caused by the co-incubation of chicken and pigeon pituitary glands with hypothalamic extract, an effect which is reversed by the DA receptor antagonist pimozide (Hall & Chadwick 1983). In the chicken, DA inhibits the release of PRL stimulated by TRH, hypothalamic extract, or by previous exposure of the pituitary gland to estrogen (Hall & Chadwick 1984). Specific DA-binding sites identified in the anterior pituitary are found to be more abundant in laying than in incubating hens (Macnamee & Sharp 1989). Moreover, in cultured turkey pituitary cells, D_2 DA receptor agonists (D_2AGs) inhibit VIP-stimulated PRL release and PRL mRNA steady-state levels (Xu *et al.* 1996). Data from *in vivo* studies suggest that DA has both stimulatory and inhibitory effects on turkey PRL secretion (Youngren *et al.* 1995, 1996*b*). The presence of both D_1 and D_2 DA receptor mRNA in the turkey brain and pituitary (Schnell *et al.* 1999, Chaiseha *et al.* 2003) suggests that DA may exhibit biphasic actions within the turkey hypothalamus and pituitary gland. DA appears to regulate PRL secretion centrally through stimulatory D_1 and inhibitory D_2 DA receptors, while at the pituitary level it exhibits an inhibitory effect on PRL secretion induced by VIP or electrical stimulation (Youngren *et al.* 1998).

DA plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to release PRL (Youngren *et al.* 1996*b*). Dynorphin, serotonin, DA and VIP all appear to stimulate avian PRL secretion along a pathway expressing κ-opioid, serotonergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (for review see El Halawani *et al.* 2000). The anatomical distribution of the avian DAergic system apparently resembles that of mammals (Moons *et al.* 1994), as DA neurons are found throughout the avian hypothalamus (Kiss & Peczely 1987, Reiner *et al.* 1994, Al-Zailaie & El Halawani 2000).

The tripeptide hypothalamic-releasing factor, TRH, and the avian PRF, VIP, produce a stimulation of PRL transcription (Camper *et al.* 1985, Yan *et al.* 1991, Tong *et al.* 1998), and DA effects a transcriptional repression of the PRL gene in cultured rat anterior cell lines (Elsholtz *et al.* 1991, Fischberg & Bancroft 1995). However, the combined effects of these factors on PRL gene

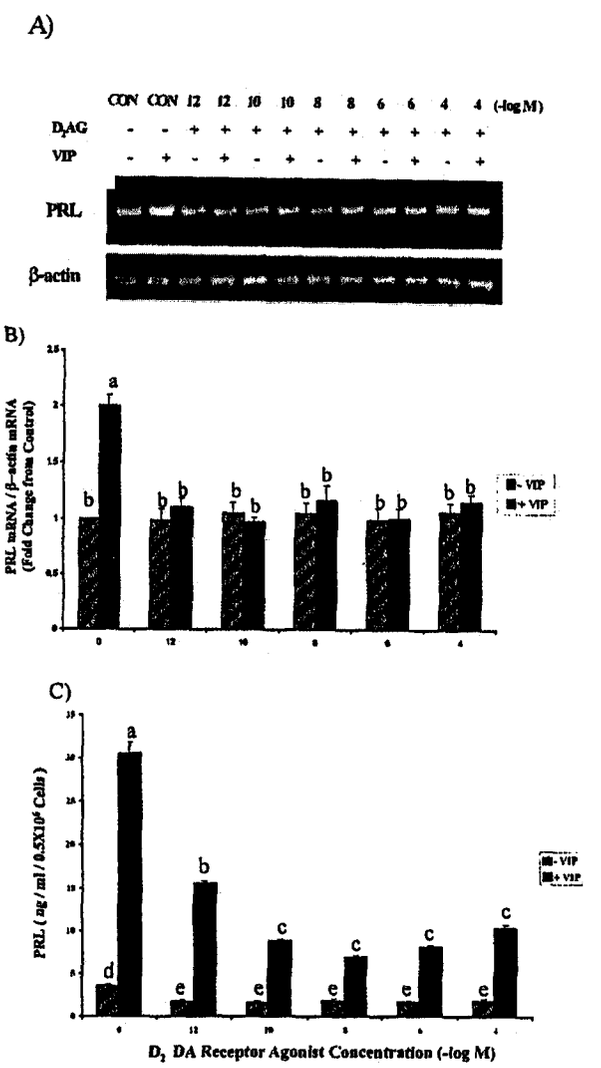


Figure 1 Dose–response effects of a D_2AG on basal and VIP-stimulated PRL mRNA expression levels and PRL release in cultured turkey anterior pituitary cells. Cultured pituitary cells ($0{\cdot}5\times10^6$ cells/treatment) were preincubated with either vehicle (control) or varying concentrations (0, 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} M) of the D_2AG R(−)-propylnorapomorphine for 30 min, followed by subsequent incubation with or without VIP (10^{-7} M) for 3 h. (A) Representative photographs of separated RT-PCR products of PRL and β-actin on ethidium bromide-stained agarose gel. (B) Relative quantification of PRL mRNA levels, which were normalized by endogenous β-actin mRNA levels. Values (means±S.E.M., n=6) of three different experiments, with two replicates per treatment were expressed as fold changes from vehicle-treated control (basal) values, which were assigned a value of 1·0. (C) Dose-dependent inhibition of basal and VIP-stimulated PRL release by the D_2AG (n=6). Significant differences (P<0·05) are identified by different letters.

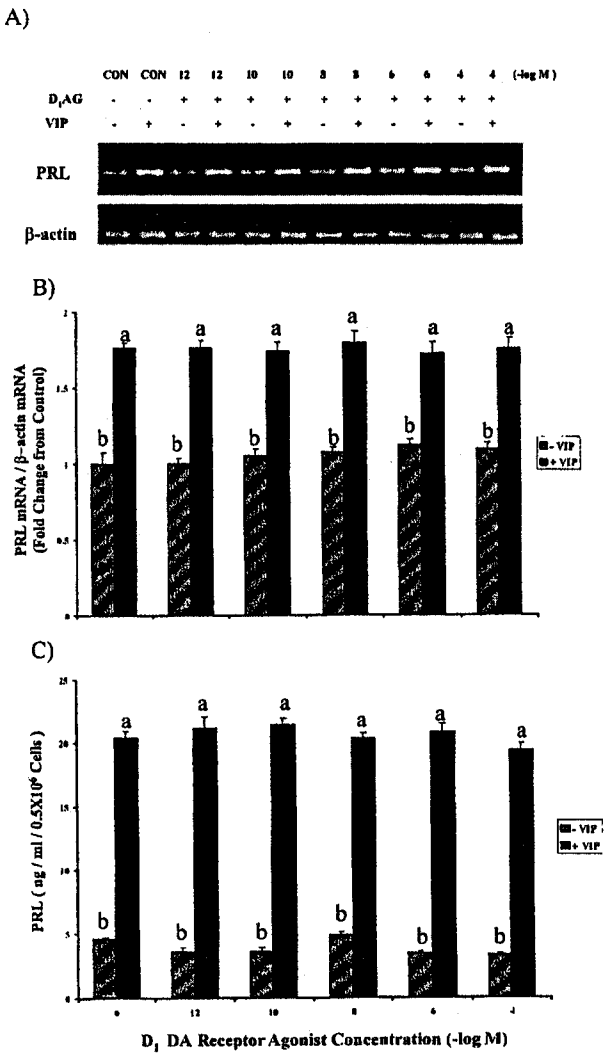


Figure 2 Dose–response effect of a D_1AG on basal and VIP-stimulated PRL mRNA expression levels and PRL release in cultured turkey anterior pituitary cells. Cultured pituitary cells were preincubated with either vehicle or varying concentrations (0, 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} M) of the D_1AG (+)-SKF 38393 for 30 min, followed by subsequent incubation with or without VIP (10^{-7} M) for 3 h. See Fig. 1 legend for details.

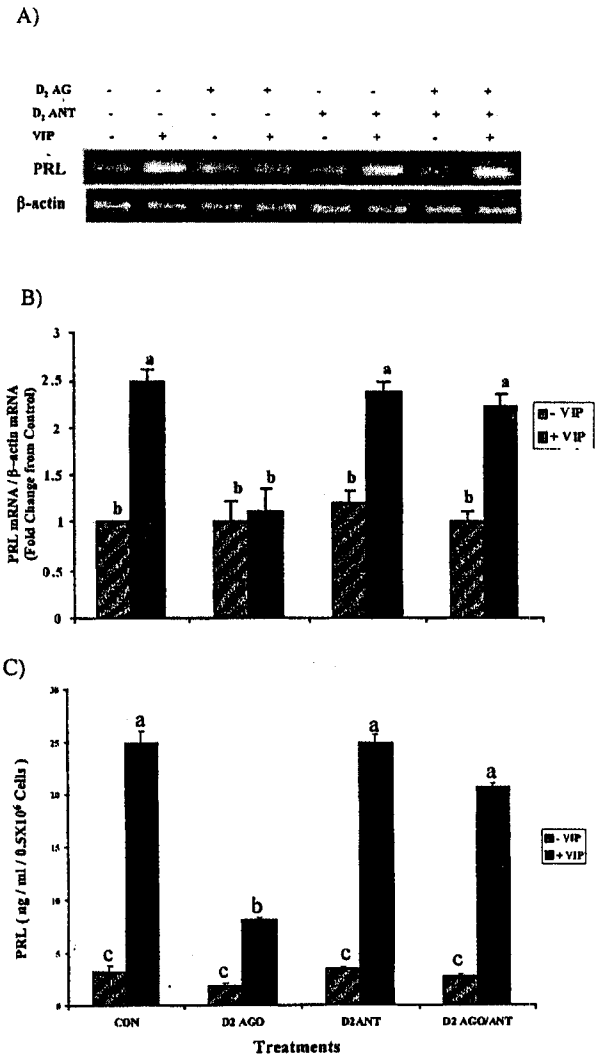
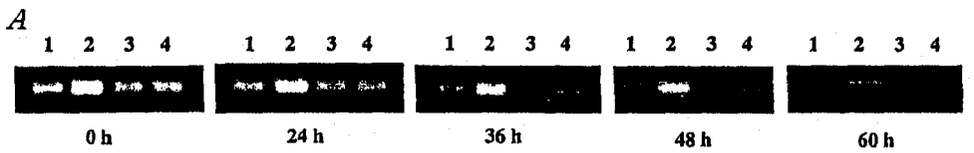
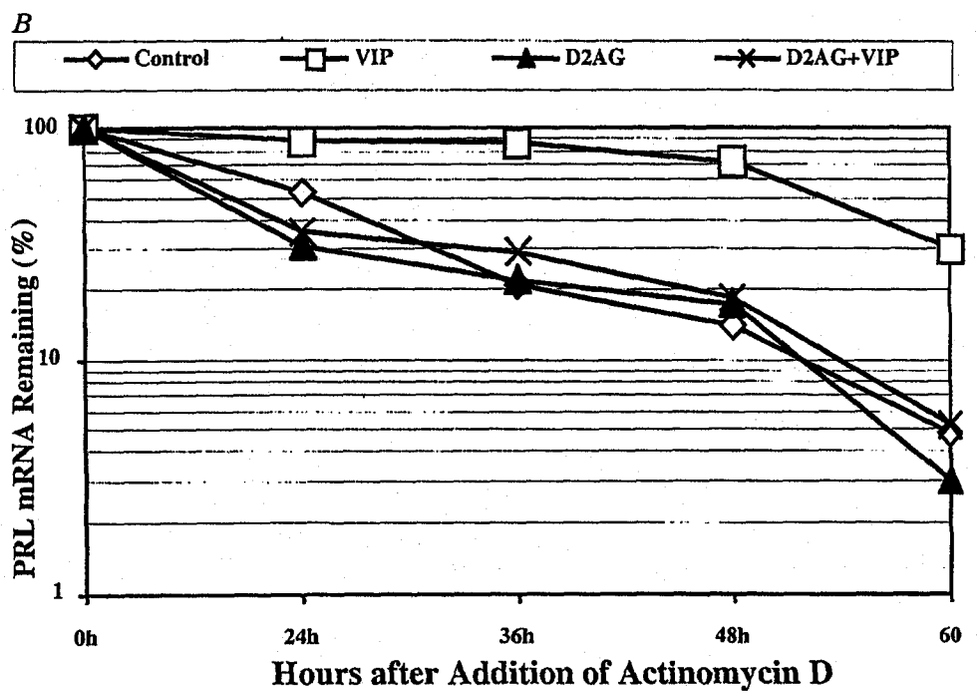


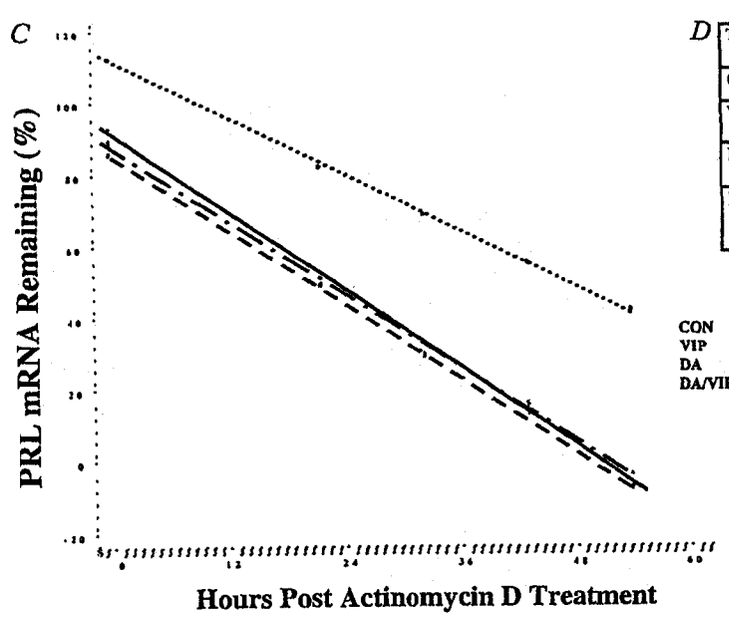
Figure 3 The specificity of the D_2AG in inhibiting basal and VIP-stimulated PRL mRNA expression levels and PRL release in cultured turkey anterior pituitary cells. Cultured pituitary cells ($0{\cdot}5\times10^6$ cells/treatment) were treated as follows: treatment 1, vehicle for 3 h and 45 min; treatment 2, vehicle for 45 min and then cVIP (10^{-7} M) was added for 3 h; treatment 3, D_2AG S(−)-eticlopride, (10^{-10} M) was added for 3 h and 45 min; treatment 4, D_2 DA receptor antagonist (10^{-10} M) for 45 min and then cVIP (10^{-7} M) was added for 3 h; treatment 5, vehicle for 15 min, then D_2AG (10^{-10} M) was added for 3 h; treatment 6, vehicle for 15 min, then D_2AG for 30 min, then cVIP for 3 h; treatment 7: D_2 DA receptor antagonist for 15 min, then D_2AG was added for 3·5 h; and treatment 8, D_2 DA receptor antagonist for 15 min and then D_2AG was added for 30 min and then cVIP for 3 h. See Fig. 1 legend for further details.

transcription remain largely unexplored. The aims of the present study were: (i) to investigate the interaction between VIP and DA in regulating PRL gene transcription rate, using the nuclear run-on (NRO) transcription assay, and (ii) to explore further the effects of VIP/DA on PRL mRNA steady-state levels, PRL mRNA stability (half-life), and PRL release from cultured turkey



1= Control
2= VIP (10^{-7} M)
3= D_2 AG
4= D_2 AG (10^{-10} M) + VIP





D

Treatment	PRL-mRNA $t_{1/2}$
CONTROL	26.3[b]
VIP	53[a]
D_2AG	23.6[b]
D_2AG+VIP	25.5[b]

primary pituitary cells. This study will attempt to provide further understanding of the interplay between VIP and DA and of their roles in the modulation of PRL secretion and gene transcription in birds.

Materials and methods

Experimental animals

Adult large white female Nicholas turkeys (weighing 10–13 kg) were used in these series of experiments. They were reared and housed on a 15 h light:9 h darkness lighting regimen in temperature-controlled (15–21 °C) floor pens, with food and water continuously available. All hens used were laying eggs (Youngren *et al.* 1996*a*). All hens were housed, handled and used in accordance with University of Minnesota Institutional Animal Care and Use Committee Guidelines.

Pituitary tissue collection and cell dispersion

Pituitary glands were collected from decapitated laying turkeys. The posterior lobes of the pituitary glands were removed, and the anterior lobes were dissociated using a modification of the trypsin/neuraminidase procedure described by Hopkins & Farquhar (1973). They were minced and replaced in Krebs–Ringer bicarbonate buffer solution (KRB, pH 7·4) supplemented with amino acids (MEM Eagle essential amino acids; BioWhittaker, Walkersville, MD, USA), 0·3 μg/ml glutamine sulfate (Sigma Chemical Co., St Louis, MO, USA), 2·5 mg/ml glucose (Sigma), 3 mg/ml BSA (fraction V, Sigma), and 100 μg/ml gentamycin sulfate (Sigma). Following mechanical shearing, pituitary fragments were digested enzymatically with 1 mg/ml trypsin (Sigma) and 2 μg/ml deoxyribonulease I (DNase I, Sigma) in KRB solution for 15 min at 37 °C in a shaking water bath. The supernatant was removed and replaced by a Ca^{2+}/Mg^{2+}-free supplemented KRB solution containing 2 mM EDTA, disodium salt (Sigma). After 5 min of incubation at 37 °C, the supernatant was removed and replaced by a Ca^{2+}/Mg^{2+}-free supplemented KRB solution containing 1 mM EDTA and 8 μg/ml neuraminidase (Sigma). After 15 min of incubation at 37 °C, the pituitary fragments were mechanically dispersed and the resultant cell suspension was filtered through 60 μm mesh Nitex gauze (Genson Scientific, Middleboro, MA, USA) to remove undigested particles. Following centrifugation at 250–500 ***g*** for 15 min, the supernatant was decanted and the cell pellet was resuspended in tissue culture medium consisting of medium M-199 (Life Technologies, Rockville, MD, USA) supplemented with 0·35 μg/ml $NaHCO_3$, 3% charcoal-stripped turkey poult serum, 3% fetal calf serum (Sigma), 4·8 μg/ml Hepes (Sigma), 1 μg/ml insulin (Sigma), 50 μg/ml gentamycin sulfate (Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 5 μg/ml amphotericin B (Sigma). Cell viability (90–95%) was determined using the trypan blue dye exclusion and cell numbers were counted in a hemocytometer. The dispersed cells were initially incubated for 72 h in siliconized Erlenmeyer flasks at 38·5 °C in humidified atmosphere (95% O_2 and 5% CO_2) before conducting the experiments. After preincubation, cultured pituitary cells were pelleted and resuspended in a serum-free M-199 medium (Life Technologies) supplemented with 0·1% BSA.

Pituitary nuclei isolation

Pituitary nuclei were isolated as previously described (Tong *et al.* 1998) with slight modifications. Briefly, pituitary cells were harvested by centrifugation and washed with diethylpyrocarbonate-treated

Figure 4 Effects of a D_2AG and VIP on PRL mRNA stability. Cultured anterior pituitary cells were incubated for 48 h with vehicle, VIP (10^{-7} M), D_2AG R(−)-propylnorapomorphine (10^{-10} M), or VIP+D_2AG. Actinomycin D (inhibitor of mRNA synthesis) was then added to a final concentration of 5 μM. Cells ($0{\cdot}5\times10^6$ cells/treatment) were harvested at 0, 24, 36, 48 and 60 h and total RNA was extracted at the indicated times and analyzed by semi-quantitative RT-PCR. Regression analysis was performed to calculate PRL mRNA half-life. Results were converted to a percentage of the time zero values. There were two experiments with three replicates each (n=6). (A) Representative photographs of separated RT-PCR products of PRL on ethidium bromide-stained agarose gel. (B) Relative quantification of PRL mRNA levels, which were normalized by β-actin mRNA levels. (C) Linear regression plots of the quantified PRL mRNA level, and (D) the calculated half-life of the PRL mRNA in each experimental condition. Significant differences are indicated by different superscripted letters.

PBS. Nuclei were isolated by incubating the cells for 5 min on ice in a lysing buffer containing 50 mM Tris–HCl, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 0·5% Nonidet P-40, followed by centrifugation at 1000 *g*. The nuclei were resuspended in a nuclear freezing buffer (50 mM Tris–HCl, pH 8·3, 5 mM $MgCl_2$, 1 mM DTT and 50% glycerol), snap frozen in liquid nitrogen, and stored at −80 °C in aliquots of 10×10^6 nuclei/100 µl until the NRO transcription assays were performed. The total cytoplasmic RNA in the supernatant was extracted by a commercial modification of the phenol–chloroform alcohol extraction method (RNeasy Mini Kit system; Qiagen Inc., Valencia, CA, USA) and stored at −80 °C until used for semi-quantitative RT-PCR analysis.

NRO transcription assay

The NRO transcription assay was performed by combining several techniques from previously described methods (Preston *et al.* 1990, Delidow *et al.* 1991). In brief, nuclei were thawed on ice. The *in vitro* transcription reaction contained 10×10^6 nuclei in a mix of transcription buffer (100 mM Tris–HCl, pH 8·0, 5 mM $MgCl_2$, 300 mM KCl, 0·5 mM each of ATP, CTP and GTP, 150 µCi [α-^{32}P]UTP (NEN Life Science, Boston, MA, USA), 10 mM DTT, and 200 U ribonuclease inhibitor (RNAsin; Promega, Madison, WI, USA)) for 45 min at 26 °C. The reaction was terminated by adding 20 U of RNase-free DNase I (Promega) at 37 °C for 15 min. The nuclei were then deproteinized by the addition of protein kinase K (200 µg/ml, Sigma) for 30 min at 37 °C. Unincorporated nucleotides were removed using the Quick Spin Columns system (Boehringer Mannheim, Indianapolis, IN, USA). Newly synthesized RNA transcripts were isolated using the total RNA isolation protocol (RNeasy Mini Kit system; Qiagen). The labeled PRL, luteinizing hormone (LH)-β and β-actin mRNA were quantified by hybridization to the PCR-synthesized turkey PRL,

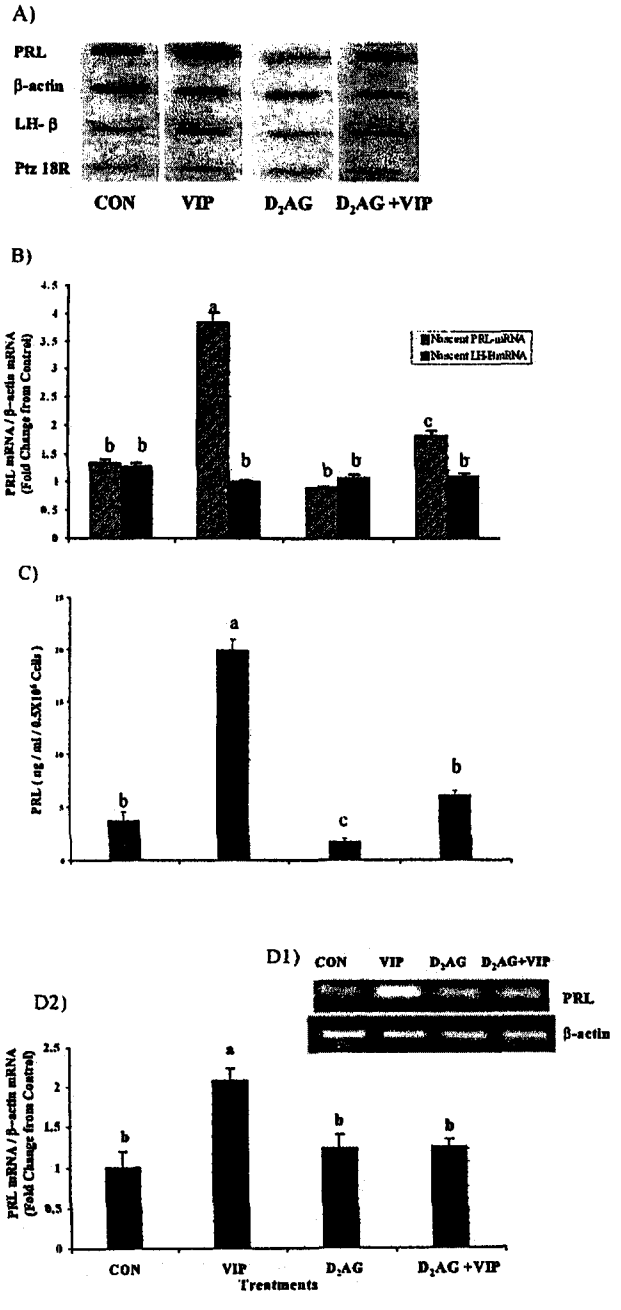


Figure 5 Effects of the D_2AG R(−)-propylnorapomorphine on basal and VIP-stimulated PRL gene transcription rate in cultured turkey anterior pituitary cells. (A) NRO transcription assay was performed on nuclei from each treatment as described, equal counts of [^{32}P]UTP-labeled nascent RNA were hybridized with PRL cDNA, β-actin cDNA, LH-β cDNA and plasmid vector (Ptz 18R) DNA. (B) Results of NRO transcription assay were normalized to β-actin after subtracting non-specific binding and were expressed as means±S.E.M. (n=5). (C) Effect of the D_2AG on VIP-stimulated PRL release in the NRO experiment. (D 1 and 2) Representative photographs of separated RT-PCR products of cytoplasmic mRNA and relative quantification of PRL mRNA levels. Columns with different letters are significantly different (P<0·05).

β-actin and LH-β cDNA, and the full-length plasmid vector (Ptz 18R), which was previously immobilized (2 μg/well) onto a Nytran membrane (ISCBioExpress, Kaysville, WT, USA) employing the described slot blot procedure (Ausubel *et al.* 1989). β-Actin cDNA and Ptz 18R vector DNA served as positive and negative controls respectively. Blots were prehybridized for 4 h at 42 °C in hybridization buffer (50% formamide, 5 × standard saline citrate (SSC), 1% SDS, 7 × Denhart's solution, 100 μg/ml salmon sperm DNA (Sigma) and 50 μg/ml yeast tRNA (Sigma)). Equal counts of radioactive elongated RNA ($0{\cdot}7–1 \times 10^6$ c.p.m.) were then added to the blots and hybridized for 72 h at 42 °C. After hybridization, the membranes were washed twice at room temperature for 15 min in 2 × SSC and 0·1% SDS, and three times at 65 °C for 20 min in 0·1 × SSC and 0·1% SDS. Membranes were then air-dried and exposed to Kodak X-ray film (Eastman Kodak, Rochester, NY, USA) at −80 °C with the use of intensifying screen to minimize the time of exposure. Autoradiographic densities were quantified using NIH Image-J software (NIH, Bethesda, MD, USA).

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from cultured pituitary cells using Trizol reagent (Gibco BRL, MD, USA) as recommended by the manufacturer with minor modifications. To reduce genomic DNA contamination, total RNA was treated with RNase-free DNase I (1 U/μg RNA) for 30 min at 37 °C. After DNA digestion, total RNA was re-extracted using the total RNA isolation protocol (RNeasy Mini Kit system) and then RNA concentration was determined using a DU Series 500 spectrophotometer (Beckman, CA, USA). Total RNA (250 ng) was reverse-transcripted using SuperScript II reverse transcriptase and Oligo $dT_{12\ 18}$ primers (Life Technologies) in a reaction volume of 20 μl containing reverse transcriptase buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8·4), 10 mM DTT, 0·5 mM of each dNTP, RNase inhibitor (RNAsin), 2·5 mM Oligo $dT_{12–18}$, and Superscript II TR (200 U), and then incubated at 42 °C for 1 h. The reaction was terminated by heating at 70 °C for 15 min. Aliquots (2 μl) of the resultant cDNA were used for the PCR reaction, which was performed in a 25 μl volume containing 200 μM of each dNTP, 50 mM KCl, 10 mM Tris–HCl, pH 8·3, 2 mM $MgCl_2$, 100 ng gene-specific sense and anti-sense primers, and 2·5 U Ampli Taq DNA polymerase (Perkin Elmer, Norwalk, CT, USA). PCR profile consisted of an initial denaturation (30 min, 94 °C) followed by cycles of denaturation (1 min, 94 °C), annealing (45 s, 60 °C), extension (1 min, 72 °C), and a final extension for one cycle (10 min, 72 °C). The number of amplification cycles was 20–22 for PRL and 30 for β-actin, which were within the linear range of amplification. Primer sequences were as follows: PRL (sense, 5′-ACC TCC TTG CCA ATC TGC TCC AGT- 3′; anti-sense, 5′-GGA GTC CTC ATC AGC GAG TTG CAG- 3′; expected size of the PCR product, 523 bp), and β-actin (sense, 5′-ACC AGT AAT TGG TAC CGG CTC CTC- 3′; anti-sense, 5′-TCT GGT GGT ACC ACA ATG TAC CCT- 3′; expected size of the PCR product, 450 bp). RT-PCR products were separated in 2% (2:1) agarose and NuSieve GTG gel (FMC Bioproducts, Rockland, ME, USA) and visualized with ethidium bromide. The gels were photographed and the intensities of the PCR products were quantified using NIH Image-J software. To correct for differences in RNA used in RT-PCR reactions, the band intensity for each RT-PCR product of PRL was normalized to that of β-actin from the same sample.

PRL RIA

Culture media were assayed for PRL content utilizing the homologous RIA described by Proudman & Opel (1981). All samples from the same experiment were assayed simultaneously. All samples from each experiment were assayed in duplicate within a single assay.

Drugs

The drugs used were: R(−)-propylnorapomorphine HCl, a D_2AG, (+)-SKF-38393, a D_1AG, and S(−)-eticlopride HCl, a D_2 DA receptor antagonist (Research Biochemicals International, Natick, MA, USA), chicken VIP (cVIP) (Peninsula Laboratory, San Carlos, CA, USA) and actinomycin D (Sigma).

Statistical analysis

The data from RIA, NRO assay and RT-PCR were analyzed using the general linear models

procedure of the Statistical Analysis system (SAS 1987). Each datapoint represents the mean ± S.E.M. of three independent experiments, with two replicates per experiment (data from NRO assays represent the means ± S.E.M. of five independent experiments). Significant differences in mean values of PRL, nascent PRL mRNA or PRL mRNA levels between treatment groups were compared using Duncan's multiple range test. $P<0{\cdot}05$ was considered statistically significant.

Results

Experiment 1: effects of D_2AG on basal and VIP-stimulated PRL mRNA levels and PRL release in turkey primary cultured pituitary cells

The D_2AG had no effect, at all concentrations used, on basal PRL mRNA steady-state levels in cultured pituitary cells after 3·5 h of incubation. On the other hand, the preincubation of pituitary cells with varying concentrations of the D_2AG for 30 min resulted in a significant inhibition of VIP-stimulated PRL mRNA levels at all concentrations used (10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}, 10^{-4} M, $P<0{\cdot}05$, Fig. 1A and B). The maximum inhibitory effect of the D_2AG was reached at a concentration of 10^{-10} M, but did not significantly differ from that observed with other concentrations. The incubation of pituitary cells with the D_2AG resulted in inhibition of basal PRL release ($P<0{\cdot}05$, Fig. 1C). Prior incubation of pituitary cells with the D_2AG at concentrations of 0, 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} M significantly inhibited VIP-stimulated PRL release in a dose-dependent manner (30·4 ± 1·3, 15·6 ± 0·4, 8·9 ± 0·1, 6·9 ± 0·2, 8·2 ± 0·1 and 10·4 ± 0·3 ng/ml respectively). The maximum inhibitory effect of VIP-stimulated PRL release was observed at the D_2AG concentration levels of 10^{-10}, 10^{-8} and 10^{-6} M.

Experiment 2: effects of a D_1AG on basal and VIP-stimulated PRL mRNA levels and PRL release in turkey primary cultured pituitary cells.

To eliminate the possibility of the involvement of the D_1 DA receptor in the inhibitory effect of the D_2AG on PRL gene expression observed in experiment 1, pituitary cells were incubated with different concentrations of the D_1AG (10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}, 10^{-4} M). Semi-quantitative RT-PCR analysis showed that the D_1AG had no effect on basal or VIP-stimulated PRL mRNA in cultured pituitary cells ($P>0{\cdot}05$, Fig. 2A and B). Basal and VIP-stimulated PRL release were not affected by the different concentrations of D_1AG added to the pituitary cells ($P>0{\cdot}05$, Fig. 2C).

Experiment 3: inhibitory effects of D_2AG on VIP-stimulated PRL mRNA levels and PRL release in turkey primary cultured pituitary cells

The D_2 DA receptor antagonist had no effect on basal or VIP-stimulated PRL mRNA levels and PRL release in pituitary cells. However, when the D_2 DA receptor antagonist was added to the cultured pituitary cells 15 min prior to the D_2AG, it significantly antagonized the inhibitory effect of the D_2AG on VIP-stimulated PRL mRNA levels ($P<0{\cdot}05$, Fig. 3A and B). Basal and VIP-stimulated PRL release were not inhibited by the D_2AG when the pituitary cells were preincubated with the D_2 DA receptor antagonist ($P>0{\cdot}05$, Fig. 3C). The D_2 DA receptor antagonist *per se* had no effect on basal or VIP-stimulated PRL release.

Experiment 4: effects of D_2AG and VIP on PRL mRNA stability in turkey primary cultured pituitary cells

The effect of VIP and/or the D_2AG on PRL mRNA stability in cultured pituitary cells was determined by measuring the half-life ($t_{1/2}$) of PRL mRNA, which was calculated by statistical regression of the data. VIP (10^{-7} M) significantly enhanced PRL mRNA stability ($t_{1/2}$=53·0 h) compared with that in non-treated control cells ($t_{1/2}$=26·3 h) and in agonist-treated cells ($t_{1/2}$= 23·6 h) ($P<0{\cdot}05$, Fig. 4A and B). Preincubating pituitary cells with the D_2AG resulted in a significant reduction in PRL mRNA half-life ($t_{1/2}$= 25·5 h) in VIP-treated pituitary cells ($P<0{\cdot}01$). The D_2AG had no effect on basal PRL mRNA $t_{1/2}$ (23·6 h). In non-actinomycin-treated control cells, PRL mRNA $t_{1/2}$ did not change throughout the experiment time course (data not shown).

Experiment 5: effects of D_2AG on basal and VIP-stimulated PRL gene transcription rate in turkey primary cultured pituitary cells

Nascent PRL mRNA increased by 3·5 ± 0·3-fold in VIP-stimulated pituitary cells in comparison with that of control cells ($P<0·05$, Fig. 5A and B). Preincubation of pituitary cells with the D_2AG significantly reduced the stimulatory effect of VIP on nascent PRL mRNA ($P<0·05$). Nascent PRL mRNA was not altered ($P>0·05$) by the treatment of pituitary cells with the agonist alone. Nascent LH-β and β-actin mRNA were not affected by the D_2AG or VIP. Similar to the nascent PRL mRNA, cytoplasmic PRL mRNA steady-state level was ~2·5-fold higher in VIP-stimulated pituitary cells than that of non-treated control cells ($P<0·05$, Fig. 5D1 and D2). The D_2AG significantly decreased the VIP stimulatory effect on PRL mRNA level ($P<0·05$). Basal and VIP-stimulated PRL release into the medium was reduced by the addition of the D_2AG to the incubating medium ($P<0·05$, Fig. 5C).

Discussion

The present results show, for the first time in birds, that DA regulates PRL synthesis in cultured primary anterior pituitary cells. DA does this, in part, by antagonizing VIP-stimulated PRL gene transcription via D_2 DA receptors. The results also show that a D_2AG reduces VIP-stimulated PRL mRNA steady-state level, PRL mRNA half-life, and PRL release at the pituitary level.

The avian PRF, VIP, is a potent stimulator of PRL release and PRL gene expression, both *in vivo* and *in vitro* (Macnamee *et al.* 1986, Opel & Proudman 1988, El Halawani *et al.* 1990*a*,*b*, Xu *et al.* 1996). Conversely, as in the present study, D_2AGs have been shown to inhibit VIP-stimulated PRL mRNA steady-state levels and PRL secretion by turkey pituitary cells (Xu *et al.* 1996). Our results show that VIP treatment increased PRL release into the medium by about 20- to 30-fold in comparison with that of untreated pituitary cells. In addition, the D_2AG, R(−)-propylnor-apomorphine, inhibited the basal and VIP-stimulated PRL release in a dose-dependent manner. However, PRL mRNA steady-state level responded differently to the action of the D_2AG. The agonist had no effect on basal PRL mRNA levels and its effect on VIP-stimulated PRL mRNA appeared to be all or none. Two possibilities may explain the differences in the PRL secretion mechanism(s) in its response to DA: (i) PRL gene expression may be more sensitive to the inhibitory effect of DA than PRL release – lower concentrations of the agonist than the ones used in the present study might show a dose–response effect, if it exists; alternatively (ii) circulating PRL levels may be fine-tuned by the relative activity of the inhibitory and stimulatory influences of DA on PRL release (Youngren *et al.* 1995, 1996*b*), since PRL release is under tonic stimulation by VIP (El Halawani *et al.* 1997). This latter possibility may also explain the findings that activation of D_2 DA receptors inhibited basal PRL release, whereas it had no effect on basal PRL mRNA levels.

Changes in PRL mRNA levels can occur because of changes in transcription and/or post-transcription events. The present results show that the D_2AG, R(−)-propylnorapomorphine, significantly inhibited the stimulatory effect of VIP on PRL mRNA at the transcriptional level. Previous studies in mammals have demonstrated the involvement of the pituitary-specific transcription factor (Pit-1, GHF-1) in the hormonal regulation of PRL transcriptional activity, including the inhibitory response to DA (Iverson *et al.* 1990, Elsholtz *et al.* 1991, Yan & Bancroft 1991). Recent work shows that the inhibition of PRL gene transcription by DA is conferred by proximal promoter sequences and that binding sites for Pit-1 are important in this response (Elsholtz *et al.* 1991, Lew *et al.* 1994). A conserved consensus Pit-1-binding site has been proposed in the avian and teleost PRL/growth hormone gene family (Ohkubo *et al.* 1996). Pit-1 cDNA has been cloned in the turkey (Wong *et al.* 1992, Kurima *et al.* 1998) and chicken (Tanaka *et al.* 1999).

These findings, taken together with the results of the present study, suggest that the inhibitory effect of DA on VIP-induced PRL gene transcription may result from DA suppression of the transactivating function of Pit-1, which is mediated by a pertussis toxin-sensitive G protein, especially $G_{o\alpha}$ or $G_{i\alpha 2}$. The major transactivating domain of mammalian Pit-1 has been localized to the N-terminal region and found to be suppressed by DA (Lew & Elsholtz 1995). Studying the structure and transcription-initiating mechanism of the chicken PRL gene indicates the involvement of Pit-1 and cAMP in the activation of PRL gene

expression (Ohkubo *et al.* 2000). However, such a suggestion must be taken with caution as the results of a recent study revealed the absence of Pit-1 protein in turkey lactotrophs (Weatherly *et al.* 2001).

The inhibition of transcription rate by DA was not the only determinant of the inhibitory effect of DA on the steady-state levels of PRL mRNA; the decay rate (half-life) was also inhibited by DA. Consistent with a previous study on turkey pituitary cells (Tong *et al.* 1998), VIP significantly increased PRL mRNA half-life from 26·3 h in control cultures to 53·0 h in VIP-treated cells, an effect that was reversed by pre-incubating the cells with the D_2AG. This is the first time that the inhibitory effect of DA on PRL mRNA stability has been demonstrated in avian or mammalian species. The molecular mechanism(s) by which activation of D_2 DA receptors destabilizes PRL mRNA half-life remains to be clarified. Alterations in one or more of the following mechanisms may be involved: (i) the length of the PRL mRNA poly (A) tail (Diamond & Goodman, 1985, Jones *et al.* 1990), (ii) the effect on the destabilizing sequence elements, such as AU-rich elements, if they exist (Shaw & Kamen 1986), or (iii) the RNA-binding proteins which play a major role in regulating the mRNA half-life (Staton *et al.* 2000).

Unlike the inhibitory effect of pituitary D_2 DA receptors, D_1 DA receptor activation showed neither a stimulatory nor inhibitory influence on PRL mRNA expression or release. These results are in agreement with earlier findings from our laboratory (Youngren *et al.* 1998). D_1 DA receptors are not found on mammalian pituitary cells (Rovescalli *et al.* 1987, Sunahara *et al.* 1991). In contrast, three D_1 DA receptor subtypes have been identified and quantified in the turkey pituitary across the reproductive cycle (Schnell *et al.* 1999, Chaiseha *et al.* 2003).

In conclusion, the present results show, for the first time in an avian species, that DA inhibits VIP-stimulated PRL mRNA at the transcriptional and post-transcriptional (PRL mRNA half-life) levels via pituitary D_2 DA receptors.

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References

Al-Zailaie K & El Halawani ME 2000 Neuroanatomical relationship between immunoreactive dopamine and vasoactive intestinal peptide neurons in the turkey hypothalamus. *Poultry Science* **79** 50.

Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seiomun JG & Struhl K 1989 *Current Protocol in Molecular Biology*. Brooklyn, NY: Greene Publishing Associates.

Ben-Jonathan N & Hnasko R 2001 Dopamine as a prolactin (PRL) inhibitor. *Endocrine Reviews* **22** 724 763.

Ben-Jonathan N, Oliver C, Weiner HJ, Mical RS & Porter JC 1977 Dopamine in hypophysial portal plasma of the rat during the estrous cycle and throughout pregnancy. *Endocrinology* **100** 452 458.

Ben-Jonathan N, Arbogast LA & Hyde JF 1989 Neuroendocrine regulation of prolactin release. *Progress in Neurobiology* **33** 399 447.

Bern HA & Nicoll CS 1968 The comparative endocrinology of prolactin. *Recent Progress in Hormone Research* **24** 681 720.

Camper SA, Yao YA & Rottman FM 1985 Hormonal regulation of the bovine prolactin promoter in rat pituitary tumor cells. *Journal of Biological Chemistry* **260** 12246 12251.

Chaiseha Y, Youngren OM, Al-Zailaie K & El Halawani ME 2003 Expression of D_1 and D_2 dopamine receptors in the hypothalamus and pituitary during the turkey reproductive cycle: colocalization with vasoactive intestinal peptide. *Neuroendocrinology* **77** 105 118.

Curlewis JD, Kusters DH, Barclay JL & Anderson ST 2002 Prolactin-releasing peptide in the ewe: cDNA cloning, mRNA distribution and effects on prolactin secretion *in vitro* and *in vivo*. *Journal of Endocrinology* **174** 45–53.

Delidow BC, Billis WM, Agarwal P & White BA 1991 Inhibition of prolactin gene transcription by transforming growth factor-beta in GH_3 cells. *Molecular Endocrinology* **5** 1716–1722.

Diamond DJ & Goodman HM 1985 Regulation of growth hormone messenger RNA synthesis by dexamethasone and triiodothyronine: transcriptional rate and mRNA stability changes in pituitary tumor cells. *Journal of Molecular Biology* **181** 41–62.

El Halawani ME, Silsby JL & Mauro LJ 1990*a* Enhanced vasoactive intestinal peptide-induced prolactin secretion from anterior pituitary cells of incubating turkeys (*Meleagris gallopavo*). *General and Comparative Endocrinology* **80** 138 145.

El Halawani ME, Silsby JL & Mauro LJ 1990*b* Vasoactive intestinal peptide is a hypothalamic prolactin releasing neuropeptide in the turkey (*Meleagris gallopavo*). *General and Comparative Endocrinology* **78** 66–73.

El Halawani ME, Youngren OM & Pitts GR 1997 Vasoactive intestinal peptide as the avian prolactin-releasing factor. In *Perspectives in Avian Endocrinology*, pp 403–416. Eds S Harvey & R Etches. Bristol: Society For Endocrinology.

El Halawani ME, Youngren OM & Chaiseha Y 2000 Neuroendocrinology of prolactin regulation in the domestic turkey. In *Avian Endocrinology*, pp 233 244. Eds A Dawson & CM Chaturvedi. New Delhi: Narosa Publishing House.

Elsholtz HP, Lew AM, Albert PR & Sundmark VC 1991 Inhibitory control of prolactin and Pit-1 gene promoters by dopamine: dual signaling pathways required for D_2 receptor-regulated expression of the prolactin gene. *Journal of Biological Chemistry* **266** 22919–22925.

Fischberg DJ & Bancroft C 1995 The D_2 receptor blocked transcription in GH3 cells and cellular pathways employed by D2A to regulate prolactin promoter activity. *Molecular and Cellular Endocrinology* **111** 129–137.

Freeman ME, Kanyicska B, Lerant A & Nagy G 2000 Prolactin: structure, function, and regulation of secretion. *Physiological Reviews* **80** 1523 1631.

Hall TR & Chadwick A 1983 Hypothalamic control of prolactin and growth hormone secretion in the pituitary gland of the pigeon and the chicken: *in vitro* studies. *General and Comparative Endocrinology* **49** 135 143.

Hall TR & Chadwick A 1984 Dopaminergic inhibition of prolactin release from pituitary glands of the domestic fowl incubated *in vitro*. *Journal of Endocrinology* **103** 63–69.

Hinuma S, Habata Y, Fujii R, Kawamata Y, Hosoya M, Fukusumi S, Kitada C, Masuo Y, Asano T, Matsumoto H *et al.* 1998 A prolactin-releasing peptide in the brain. *Nature* **393** 272–276.

Hopkins CR & Farquhar MG 1973 Hormone secretion by cells dissociated from rat anterior pituitaries. *Journal of Cell Biology* **59** 277–303.

Iverson RA, Day KH, d'Emden M, Day RN & Maurer RA 1990 Clustered point mutation analysis of the rat prolactin promoter. *Molecular Endocrinology* **4** 1564–1571.

Jones PM, Burrin JM, Ghatei MA, O'Halloran DJ, Legon S & Bloom SR 1990 The influence of thyroid hormone status on the hypothalamo–hypophyseal growth hormone axis. *Endocrinology* **126** 1374–1379.

Kanasaki H, Yonehara T, Yamamoto H, Takeuchi Y, Fukunaga K, Takahashi K, Miyazaki K & Miyamoto E 2002 Differential regulation of pituitary hormone secretion and gene expression by thyrotropin-releasing hormone. A role for mitogen-activated protein kinase signaling cascade in rat pituitary GH3 cells. *Biology of Reproduction* **67** 107–113.

Kiss JZ & Peczely P 1987 Distribution of tyrosine-hydroxylase (TH)-immunoreactive neurons in the diencephalon of the pigeon (*Columba livia domestica*). *Journal of Comparative Neurology* **257** 333–346.

Kragt CL & Meites J 1965 Stimulation of pigeon pituitary prolactin release by pigeon hypothalamic extract *in vitro*. *Endocrinology* **76** 1169–1176.

Kurima K, Weatherly KL, Sharova L & Wong EA 1998 Synthesis of turkey Pit-1 mRNA variants by alternative splicing and transcription initiation. *DNA and Cell Biology* **17** 93–103.

Lew AM & Elsholtz HP 1995 A dopamine-responsive domain in the N-terminal sequence of Pit 1: transcriptional inhibition in endocrine cell types. *Journal of Biological Chemistry* **270** 7156–7160.

Lew AM, Yao H & Elsholtz HP 1994 G(i) alpha 2- and G(o) alpha-mediated signaling in the Pit-1-dependent inhibition of the prolactin gene promoter: control of transcription by dopamine D2 receptors. *Journal of Biological Chemistry* **269** 12007–12013.

Macnamee MC & Sharp PJ 1989 The functional activity of hypothalamic dopamine in broody bantam hens. *Journal of Endocrinology* **121** 67–74.

Macnamee MC, Sharp PJ, Lea RW, Sterling RJ & Harvey S 1986 Evidence that vasoactive intestinal polypeptide is a physiological prolactin-releasing factor in the bantam hen. *General and Comparative Endocrinology* **62** 470–478.

Moons L, van Gils J, Ghijsels E & Vandesande F 1994 Immunocytochemical localization of L-dopa and dopamine in the brain of the chicken (*Gallus domesticus*). *Journal of Comparative Neurology* **346** 97–118.

Ohkubo T, Araki M, Tanaka M, Sudo S & Nakashima K 1996 Molecular cloning and characterization of the yellowtail GH gene and its promoter: a consensus sequence for teleost and avian Pit-1/GHF-1 binding sites. *Journal of Molecular Endocrinology* **16** 63–72.

Ohkubo T, Tanaka M & Nakashima K 2000 Molecular cloning of the chicken prolactin gene and activation by Pit-1 and cAMP-induced factor in GH_3 cells. *General and Comparative Endocrinology* **119** 208–216.

Opel H & Proudman JA 1988 Stimulation of prolactin release in turkeys by vasoactive intestinal peptide. *Proceedings of the Society for Experimental Biology and Medicine* **187** 455–460.

Pasqualini C, Bojda F, Gaudoux F, Guibert B, Leviel V, Teissier E, Rips R & Kerdelhue B 1988 Changes in tuberoinfundibular dopaminergic neuron activity during the rat estrous cycle in relation to the prolactin surge: alteration by a mammary carcinogen. *Neuroendocrinology* **48** 320–327.

Preston GM, Billis WM & White BA 1990 Transcriptional and posttranscriptional regulation of the rat prolactin gene by calcium. *Molecular Cell Biology* **10** 442–448.

Proudman JA & Opel H 1981 Turkey prolactin: validation of radioimmunoassay and measurement of changes associated with broodiness. *Biology of Reproduction* **25** 573–580.

Reiner A, Karle EJ, Anderson KD & Medina L 1994 Catecholaminergic perikarya and fibers in the avian nervous system. In *Phylogeny and Development of Catecholamine Systems in CNS of Vertebrates*, pp 135–181. Eds WJAJ Smeets & A Reiner. Cambridge: Cambridge University Press.

Rovescalli AC, Brunello N, Monopoli A, Ongini E & Racagni G 1987 Absence of [^{3}H]SCH 23390 specific binding sites in anterior pituitary: dissociation from effects on prolactin secretion. *European Journal of Pharmacology* **135** 129–136.

SAS 1987 *User's Guide: Version 7*. Cary, NC: SAS Institute.

Schnell SA, You S & El Halawani ME 1999 D_1 and D_2 dopamine receptor messenger ribonucleic acid in brain and pituitary during the reproductive cycle of the turkey hen. *Biology of Reproduction* **60** 1378–1383.

Shaw G & Kamen RA 1986 Conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46** 659–667.

Staton JM, Thomson AM & Leedman PJ 2000 Hormonal regulation of mRNA stability and RNA-protein interactions in the pituitary. *Journal of Molecular Endocrinology* **25** 17–34.

Sunahara RK, Guan HC, O'Dowd BF, Seeman P, Laurier LG, Ng G, George SR, Torchia J, Van Tol HHM & Niznik HB 1991 Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1. *Nature* **350** 614–619.

Tanaka M, Yamamoto I, Ohkubo T, Wakita M, Hoshino S & Nakashima K 1999 cDNA cloning and developmental alterations in gene expression of the two Pit-1/GHF-1 transcription factors in the chicken pituitary. *General and Comparative Endocrinology* **114** 441–448.

Taylor MM & Samson WK 2001 The prolactin releasing peptides: RF-amide peptides. *Cellular and Molecular Life Sciences* **58** 1206–1215.

Tong Z, Pitts GR, You S, Foster DN & El Halawani ME 1998 Vasoactive intestinal peptide stimulates turkey prolactin gene expression by increasing transcription rate and enhancing mRNA stability. *Journal of Molecular Endocrinology* **21** 259–266.

Weatherly KL, Ramesh R, Strange H, Waite KL, Storrie B, Proudman JA & Wong EA 2001 The turkey transcription factor Pit-1/GHF-1 can activate the turkey prolactin and growth hormone gene promoters in vitro but is not detectable in lactotrophs *in vivo*. *General and Comparative Endocrinology* **123** 244–253.

Wong EA, Silsby JL & El Halawani ME 1992 Complementary DNA cloning and expression of Pit-1/GHF-1 from the domestic turkey. *DNA and Cell Biology* **11** 651–660.

Xu M, Proudman JA, Pitts GR, Wong EA, Foster DN & El Halawani ME 1996 Vasoactive intestinal peptide stimulates prolactin mRNA expression in turkey pituitary cells: effects of dopaminergic drugs. *Proceedings of the Society for Experimental Biology and Medicine* **212** 52–62.

Yan GZ & Bancroft C 1991 Mediation by calcium of thyrotropin-releasing hormone action on the prolactin promoter via transcription factor pit-1. *Molecular Endocrinology* **5** 1488–1497.

Yan GZ, Pan WT & Bancroft C 1991 Thyrotropin-releasing hormone action on the prolactin promoter is mediated by the POU protein Pit-1. *Molecular Endocrinology* **5** 535–541.

Youngren OM, Pitts GR, Phillips RE & El Halawani ME 1995 The stimulatory and inhibitory effects of dopamine on prolactin secretion in the turkey. *General and Comparative Endocrinology* **98** 111–117.

Youngren OM, Chaiseha Y, Phillips RE & El Halawani ME 1996*a* Vasoactive intestinal peptide concentrations in turkey hypophyseal portal blood differ across the reproductive cycle. *General and Comparative Endocrinology* **103** 323–330.

Youngren OM, Pitts GR, Phillips RE & El Halawani ME 1996*b* Dopaminergic control of prolactin secretion in the turkey. *General and Comparative Endocrinology* **104** 225–230.

Youngren OM, Chaiseha Y & El Halawani ME 1998 Regulation of prolactin secretion by dopamine and vasoactive intestinal peptide at the level of the pituitary in the turkey. *Neuroendocrinology* **68** 319–325.

Yuan ZF & Pan JT 2002 Involvement of angiotensin II, TRH and prolactin-releasing peptide in the estrogen-induced afternoon prolactin surge in female rats: studies using antisense technology. *Life Sciences* **71** 899–910.

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