Vasoactive intestinal peptide stimulates turkey prolactin
gene expression by increasing transcription rate and
enhancing mRNA stability

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ABSTRACT

This study evaluates the transcriptional and post-transcriptional regulation of prolactin (PRL) by vasoactive intestinal peptide (VIP). Pituitary nuclei from laying (control), incubating (with enhanced VIP secretion), and VIP-immunized laying turkey hens, and from pituitary cells cultured with or without VIP were used in nuclear run-on transcription assays. Cytoplasmic PRL mRNA was analyzed by slot blot hybridization. PRL transcription was greater in hyperprolactinemic incubating birds (PRL/β-actin=3·33) than in laying birds (PRL/β-actin=1·83). VIP-immunoneutralized birds had 47% and 51% decreases in PRL transcription and cytoplasmic PRL mRNA, respectively when compared with laying birds. In primary pituitary cell cultures, VIP significantly increased the transcription rate of PRL (3·8-fold) and cytoplasmic PRL mRNA (3·2-fold) compared with that of non-VIP-treated pituitary cells. The stability of pre-existing PRL mRNA was measured by Northern blot analysis after addition of actinomycin D. PRL mRNA half-lives were calculated using a two-component model, with a first-long component of 18·0±1·0 h and a second-short component of 3·7±0·7 h in non-VIP-treated pituitary cells. Both half-lives were significantly increased (53·2±6·9 and 26·3±4·3 h) in VIP-treated cells. The present data show that VIP acts to stimulate PRL expression by up-regulating the transcription rate of PRL and by enhancing PRL mRNA stability.

INTRODUCTION

Vasoactive intestinal peptide (VIP), a 28-amino acid peptide, was first isolated from porcine duodenum (Said & Mutt 1970). Subsequently, it has been found to be widely distributed in the central and peripheral nervous systems, and is believed to function as a neurotransmitter and neuroendocrine substance (Said & Rosenberg 1976, Larsson et al. 1976, Rosselin et al. 1982). VIP is involved in the regulation of the secretion of mammalian prolactin (PRL) (Krao et al. 1978, Samson et al. 1980, Parisi et al. 1990), but it is not the main regulator of PRL release, as PRL is under tonic inhibitory dopaminergic control by the hypothalamus (Talwalker et al. 1963, Kamberi et al. 1971, Lamberts & Macleod 1990). In contrast, several lines of evidence indicate that PRL release is under tonic stimulatory control by the avian hypothalamus, exerted through VIP (El Halawani et al. 1997). VIP-immunoreactive cells have been localized within the mediobasal hypothalamus and external median eminence in several avian species (Yamada & Mikami 1982, Macnamee et al. 1986, Peczely & Kiss 1988, Mauro et al. 1989). Changes in the number and size of VIP immunoreactive cells have been demonstrated to parallel plasma concentrations of PRL in chickens (Sharp et al. 1989), turkeys (Mauro et al. 1989) and doves (Cloues et al. 1990). Hypothalamic VIP content, VIP mRNA abundance and the affinity of anterior pituitary VIP-binding sites have been shown to be positively correlated with the concentration of plasma PRL in turkeys (Mauro et al. 1992, Rozenboim & El Halawani 1993, You et al. 1995). Furthermore, in vivo administration of VIP has been shown to increase plasma PRL concentrations in ring doves (Lea & Vowles 1986), chickens (Macnamee et al. 1986, Talbot et al. 1991) and turkeys (Pitts et al. 1994a). VIP also has been shown to stimulate PRL release in turkeys in vitro.
VIP immunoneutralization was shown to suppress plasma PRL and terminate incubation behavior in chickens (Sharp et al. 1989) and turkeys (El Halawani et al. 1995), and to prevent the development of crop-sac tissue in incubating ring doves (Lea et al. 1991).

In addition to its PRL-releasing ability, VIP stimulates PRL gene expression in avian species. Administration of VIP into chickens and turkeys increased pituitary PRL mRNA in vivo (Talbot et al. 1991, Pitts et al. 1994b) and in vitro (Kansaku et al. 1995, Xu et al. 1996). Passive immunization of incubating chickens with anti-VIP serum or active immunization against VIP in turkeys decreased the pituitary content of PRL mRNA (Talbot et al. 1991, Youngren et al. 1994). PRL gene expression has been shown to be regulated at the transcriptional level by dopamine (Elsholtz et al. 1991) and thyrotropin-releasing hormone (TRH, Benker et al. 1990) in mammals. TRH has also been demonstrated to act post-transcriptionally. It is not clear how VIP increases PRL expression in avian species. The present study was designed to examine the hypothesis that VIP up-regulates pituitary PRL mRNA at both transcriptional and post-transcriptional levels in turkeys.

MATERIALS AND METHODS

Animals

Adult large white female Nicholas turkeys were divided into two groups according to reproductive status: incubating and laying birds.

Immunization

Turkey VIP (Microchemical Laboratory, University of Minnesota) was conjugated to keyhole limpet hemocyanin (KLH) according to the method described by Lerner et al. (1981). The first dose of immunogen contained 125 μg KLH–VIP suspended in 0.25 ml Freund’s complete adjuvant which was subsequently brought to 0.5 ml with saline. The mixture was emulsified and injected intradermally around the neck. Subsequent boosters were given with 25 μg KLH–VIP in Freund’s incomplete adjuvant. Control turkeys were immunized only with Freund’s adjuvant. Immunizations were given on the day of photostimulation and were repeated three times at 4-week intervals. Birds were killed on the 14th day after the last immunization. There were six birds in each treatment group.

Cell culture and treatment conditions

Anterior pituitary cells from immunized turkeys were dissociated using the procedure described by Fehrer et al. (1985), and cultured in M-199 medium (Life Technologies Inc., Grand Island, NY, USA), containing 3% charcoal-stripped poult serum, 3% fetal bovine serum, 0.05 mg/ml gentamycin, 0.1 mg/ml penicillin/streptomycin, 5 μg/ml amphotericin, 0.1% BSA and with or without turkey VIP (1 × 10⁻⁷ M) at 39.5 °C in a 95% O₂ and 5% CO₂ incubator. The culture medium was replaced every 24 h.

To examine the effects of VIP on PRL expression, cell cultures were terminated after 72 h. Cells (1 × 10⁶) from each treatment were washed twice with cold PBS and then used for nuclear isolation. Three independent experiments with two replicates per treatment were performed.

For determination of PRL mRNA stability, the culture medium was replaced with fresh medium plus 5 μg/ml of the RNA synthesis inhibitor, actinomycin D, 48 h after initial incubation. Cells (1 × 10⁶) from each treatment were harvested 0, 24, 36, 48, and 60 h after the addition of actinomycin D. Thereafter, cells were washed twice with cold PBS and then stored at −80 °C until RNA was isolated. Three independent experiments were conducted. There were two replicates at each time period in each experiment.

Nuclear run-on transcription assay

Nuclei from pituitaries or pituitary cells were isolated and used for nuclear run-on transcription assays as described by Tong et al. (1997). Briefly, each transcription reaction contained 150 μCi α-³²P-UTP, 10–12 × 10⁶ nuclei, 10 mM ATP, GTP, CTP and transcription buffer. Labeled RNA was extracted with RNA stat-50 LS (Tel-Test, Friendwood, TX, USA). RNA was precipitated with ethanol, pelleted by centrifugation at 12,000 g for 20 min and was washed twice with 1 M NH₄OAc in ethanol to remove unincorporated α-³²P-UTP. Slot blots were prepared (Ausubel et al. 1989) using linearized, denatured turkey PRL cDNA, control plasmid DNA (ptz18R), control β-actin cDNA and with or without luteinizing hormone (LH) β-subunit cDNA. Equal counts of each RNA sample were hybridized to DNA immobilized on slot blots that had been prehybridized for 7 h. After hybridization for 72 h at 42 °C, filters were washed under increasingly stringent conditions, with the final wash condition of 0.1 × SSC, 0.5% SDS, 15 min, 65 °C. After washing, filters were exposed to X-ray film for 14 days at

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Slot blot analysis
Cytoplasmic RNA was prepared from cell lysates after removal of nuclei by centrifugation through a sucrose gradient as described by O’Connor & Wade (1992). RNA was extracted with phenol–chloroform–isoamyl alcohol, precipitated with ethanol and pelleted by centrifugation at 12 000 g, then washed twice with 70% ethanol, resuspended in dimethyl pyrocarbonate-treated water, and stored at −80 °C until used for slot blot analysis (Sambrook et al. 1989). One microgram cytoplasmic RNA from each sample was immobilized on a nitrocellulose membrane in duplicate using a slot blot manifold (Scheicher & Schuell Inc., Keene, NH, USA). PRL mRNA was quantified by hybridization to a nick-translated turkey PRL cDNA probe (Wong et al. 1991). The membrane was prehybridized for 5 h, then hybridized at 42 °C for 16 h, followed by washing once with 2 × SSC for 5 min at room temperature, twice with 2 × SSC and 1% SDS for 30 min at 65 °C, once with 0·1 × SSC and 1% SDS for 30 min at room temperature. After washing, the membrane was exposed to X-ray film for 20 h at −80 °C. The film was then analyzed by densitometric scanning.

Northern blot analysis
The half-life of PRL mRNA was determined by measuring the decay of pre-existing mRNA using Northern blot.
Total RNA from 1 × 10⁶ pituitary cells was isolated according the methods of Chomczynski & Sacchi (1987). The RNA was fractionated on a 1% agarose–formaldehyde gel by electrophoresis and transferred to Gene Screen (Dupont, Boston, MA, USA) with 0·025 M sodium phosphate solution (pH 6·5). The RNA was then immobilized using a vacuum oven at 80 °C for 2 h. Blots were hybridized to 32P-labeled turkey PRL cDNA. After hybridization, membranes were washed and exposed to X-ray film using essentially the same conditions as described above for slot blot analysis. The autoradiographic films were quantified by densitometric scanning.

Statistical analysis
Nuclear run-on data were analyzed using a complete block design. Northern blot data at various times were converted to percent of the time zero value. The actinomycin D data were interpreted as a two-component model. Accordingly, the PRL mRNA decay was analyzed as two-component disappearance curves (Norman & Fortier 1970). Half-lives were calculated using linear regression analysis. Means were compared using Tukey’s Studentized Range Test within the General Linear Models Procedure of the Statistical Analysis System (SAS Inst. 1989). A P value less than 0·05 was considered statistically significant. Results are expressed as means ± s.e.m.

RESULTS

Effects of VIP immunization on PRL expression
Pituitary cytoplasmic steady-state PRL mRNA content was significantly greater in incubating birds than in laying birds (1·52-fold, P<0·05). When laying birds were immunized against endogenous VIP, pituitary cytoplasmic PRL mRNA content was significantly reduced by 51%, (P<0·05; Fig. 1). Similar results were observed for PRL transcription (Fig. 2). PRL transcription was greater in hyperprolactinemic incubating birds (PRL/β-actin=3·33) than in laying control birds (PRL/β-actin=1·83). VIP immunoneutralization decreased PRL transcription by 47% in comparison with that of laying control birds (P<0·05; Fig. 2). The transcription of LH β-subunit did not significantly change among incubating, laying and VIP-immunized laying birds, indicating that the changes in PRL transcription were specific to that hormone.

Effects of VIP on PRL expression
The effect on cytoplasmic PRL mRNA abundance of incubating pituitary cells with VIP for 72 h is shown in Fig. 3. Cytoplasmic PRL mRNA content was 3·2-fold greater in pituitary cells treated with 1 × 10⁻⁷ M VIP than in primary pituitary cell cultures not treated with VIP (P<0·05). Similarly, the rate of PRL transcription was increased 3·85-fold in VIP-treated pituitary cells as compared with untreated control cells (P<0·05, Fig. 4).

The half-life (t₁/₂) of PRL mRNA was determined by measuring the decay of pre-existing mRNA by Northern blot analysis after incubating with actinomycin D. The half-life of PRL mRNA was calculated using logarithmically transformed data. As shown in Fig. 5, the degradation of PRL mRNA was suppressed by VIP in cultured anterior pituitary cells. During the first 24 h in the presence of actinomycin D, the concentrations of PRL mRNA decreased to 72·3 ± 5·3% in control pituitary
cells, compared with 88.9 ± 2.7% in VIP-treated cells. At 36 and 48 h, PRL mRNA concentrations were 22.7 ± 3.5% and 4.2 ± 0.9% of those at time zero respectively, in the presence of the transcription inhibitor alone, in contrast to their corresponding values of 77.6 ± 5.8% and 54.4 ± 8.5% in VIP-treated cells.

To calculate PRL mRNA half-lives, it was determined that the data were best fitted to a two-component model, with a first-long half-life of 18.0 ± 1.0 h and a second-short half-life of 3.7 ± 0.7 h for PRL mRNA in non-VIP-treated cells. However, first and second half-lives of 53.2 ± 6.9 h and 26.3 ± 4.3 h respectively, were calculated for PRL mRNA in VIP-treated pituitary cells.

**DISCUSSION**

The results of the present study demonstrate for the first time in birds that VIP, the avian prolactin-releasing factor, regulates PRL gene expression by acting both at the transcriptional level and at the level of mRNA stability.

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Previously, high levels of PRL expression were observed in response to exogenous VIP (Talbot et al. 1991, Pitts et al. 1994b, Xu et al. 1996) and under conditions of an enhanced VIP secretion (Wong et al. 1991, Youngren et al. 1996). In contrast, VIP immunoneutralization was shown to suppress PRL mRNA steady-state concentrations (Youngren et al. 1994). The present results indicate a strong stimulation of PRL transcription rate by VIP, which is consistent with the increased steady-state concentrations of PRL mRNA. Conversely, immunoneutralization of endogenous turkey VIP is accompanied by a significant reduction of PRL transcription and steady-state values of PRL mRNA. In mammals, PRL expression is transcriptionally regulated by dopamine (Maurer 1981, Shull & Gorski 1989) and TRH (Laverriere et al. 1983).

The intracellular mechanisms that regulate PRL transcription by VIP are presently unknown. Previous studies have shown that VIP causes the accumulation of intracellular cAMP in mammalian pituitary cells (Onali et al. 1983, Guild & Drummond 1984, Bjoro et al. 1990). Although direct evidence for cAMP accumulation by VIP in the avian pituitary cells has not been provided, protein kinase C has been shown to mediate VIP-induced PRL secretion and gene expression in cultured turkey anterior pituitary cells (Sun &
pituitary cell cultures treated with VIP compared with untreated cell cultures. These results suggest that VIP increases the steady-state PRL mRNA concentration, in part, by enhancing mRNA stability. Our findings show that, after an initial slow rate of decrease, the rate of decay of PRL mRNA is significantly faster after adding actinomycin D alone \( (t_{1/2}=3.7 \pm 0.7 \text{ h}) \) than after treatment with VIP and actinomycin D \( (t_{1/2}=26.3 \pm 4.3 \text{ h}) \). These findings are consistent with our earlier data that showed the half-life of PRL mRNA to be positively correlated with PRL mRNA abundance and VIP release (Youngren et al. 1996, Tong et al. 1997). Thus, in hyperprolactinemic incubating hens, which have up-regulated steady-state concentrations of PRL mRNA (Wong et al. 1992), the half-life of PRL mRNA is longer compared with that of reproductively inactive hypoprolactinemic hens which display reduced PRL mRNA abundance and VIP release (Youngren et al. 1996).

The mechanism(s) of the VIP-induced increase in PRL mRNA half-life is not known. Cytoplasmic mRNAs are usually associated with RNA binding proteins that can regulate mRNA stability (Sani et al. 1990). The 3'-untranslated region of mRNA with an AU-rich domain has been shown to destabilize or stabilize mRNA transcripts (Bickel et al. 1990, Iwai et al. 1991). Analysis of the turkey PRL mRNA has revealed the presence of such an AU-rich domain (Wong et al. 1991, Kurima et al. 1995). Furthermore, activation of protein kinase C has been shown to stabilize mRNA transcripts by acting on an AU-rich domain in the 3'-untranslated region (Iwai et al. 1991). Therefore, it is possible that VIP modifies and activates some intracellular proteins via a second messenger system that interacts with the 3'-untranslated region to protect PRL mRNA from degradation. The finding in the present study, that PRL mRNA first decayed at a slow rate and thereafter decayed faster after the addition of actinomycin D to pituitary cells, also suggests the presence of a protein(s) that could mediate the stability of PRL mRNA. Stimulation of PRL expression mediated by a change in half-life of PRL mRNA could serve as an important biological mechanism for rapidly and economically regulating PRL concentrations in order to reflect changes in specific reproductive conditions.

In conclusion, the present study provides strong evidence that VIP, the avian PRL-releasing factor, has an important role in the regulation of PRL gene expression in avian species. VIP increases PRL mRNA concentrations by acting both at the transcriptional level and at the level of PRL mRNA stability.

FIGURE 5. Effect of VIP on PRL mRNA degradation. Anterior pituitary cells were incubated with or without VIP for 48 h before actinomycin D was added. Cells \( (1 \times 10^6) \) were harvested at various times, as indicated above, for RNA isolation. PRL mRNA was measured by Northern blot hybridization to PRL cDNA. Results were converted to a percentage of the time zero values by logarithmically transformed. Data points represent the mean ± s.e.m. of three independent experiments. In most instances the s.e.m. is too small to be seen.

El Halawani 1995). Tumor-promoting phorbol ester, a protein kinase C agonist, has also been shown to increase adenylate cyclase activity and cAMP in birds (Johnson & Tilly 1991) and to stimulate PRL gene transcription (Murdoch et al. 1985). Forskolin, which increases intracellular cAMP, was also shown to stimulate PRL expression in chickens (Kansaku et al. 1995). Therefore, it is most likely that VIP up-regulates PRL transcription by altering the activation of transcription factor through second messenger systems.

It has become increasingly apparent that regulation of mRNA stability is an important mechanism by which releasing factors affect hormone gene expression (Nielsen & Shapiro 1990). Several methods are available for measuring mRNA half-life, such as pulse-chase analysis and Northern blot analysis in the presence of a transcriptional inhibitor, actinomycin D (Harrold et al. 1991). The latter appears to be the more convenient and widely used method for \textit{in vitro} studies. In addition, the mRNA half-life measured using actinomycin D is comparable to that obtained with pulse-chase analysis (Harrold et al. 1991). Although actinomycin D is considered to disturb cellular metabolism and to block synthesis of labile proteins that regulate mRNA stability (Kilen et al. 1996), the cell morphology did not change in the present study during exposure to actinomycin D. In the present study, the PRL mRNA half-life was measured using Northern blot analysis in the presence of actinomycin D. We found that the half-life of PRL mRNA was significantly \( (P<0.05) \) increased in

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