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General and Comparative Endocrinology 142 (2005) 267–273
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

The relative importance of vasoactive intestinal peptide and peptide histidine isoleucine as physiological regulators of prolactin in the domestic turkey ☆

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Received 3 September 2004; revised 1 December 2004; accepted 3 December 2004
Available online 1 April 2005

Abstract

In mammals, prolactin (PRL) secretion is regulated by vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI). In birds, however, VIP is considered a PRL-releasing factor (PRF), while the role of PHI is unknown. The purpose of this study was to compare the effects of turkey PHI (tPHI) and turkey VIP (tVIP) on PRL secretion in vitro, and to study their physiological significance in vivo through active immunization against tPHI and tVIP. In vitro studies were conducted using pituitary cell cultures from female turkeys. In the in vivo study, female turkeys were immunized with keyhole limpet hemocyanin (KLH; control), synthetic tPHI conjugate (KLH–tPHI), or synthetic tVIP conjugate (KLH–tVIP). Both tVIP and tPHI stimulated PRL secretion from anterior pituitary cells in a dose response manner. However, tPHI was 100-fold less potent than tVIP in stimulating maximum PRL secretion in vitro. In addition, the highest dose (10^{-4} M) of tPHI inhibited its own PRL-releasing activity as well as that of VIP-stimulated PRL release. Whereas, circulating PRL levels and nesting activity remained low and unchanged during the photo-induced reproductive cycle (i.e., experimental period) in tVIP-immunized birds, control and tPHI-immunized turkeys showed a significant increase in plasma PRL levels and in the incidence of incubation behavior over time following photostimulation. These findings, taken together with earlier results, indicate that VIP is the sole physiological PRF in the turkey (avian species).

Keywords: Avian; Immunization; Peptide histidine isoleucine; Prolactin; Turkey; Vasoactive intestinal peptide

1. Introduction

The prepro-vasoactive intestinal peptide (VIP) gene also encodes a VIP-related peptide (48% homology), 27 amino acid peptide histidine isoleucine amide (PHI) in turkeys (You et al., 1995) and in rats (Nishizawa et al., 1985) or peptide histidine methionine (PHM) in humans (Itoh et al., 1983). The sequences encoding VIP and PHI are contained in two separate exons in mammals (Bodner et al., 1985; Gozes et al., 1986; Lamperti et al., 1991). In rats, PHI and VIP have an equally potent stimulatory effect on prolactin (PRL) secretion (Kaji et al., 1984; Ohta et al., 1985; Samson et al., 1983; Werner et al., 1983). PHI/PHM distribution is identical to that of VIP, is found in many organs, and co-exists in the same neural elements (Christofides et al., 1982, 1984; Fahrenkrug and Pedersen, 1986; Fahrenkrug, 1987). PHI exhibits preferential binding to VIP receptors in rat tissues, indicating that VIP and PHI mediate their physiological

☆ This research was supported by BARD #US-3399-03C.
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0016-6480/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2004.12.024

effects through common receptors (Huang and Rorstad, 1990). Passive immunization against VIP and PHI significantly blunts PRL secretion in serotonin (5-HT)-stimulated (Kaji et al., 1985a) and ether-induced rats (Kaji et al., 1985b).

Unlike mammals, the prepro-VIP mRNA is alternatively spliced in a tissue-specific manner in birds (Talbot et al., 1995; You et al., 1995). VIP mRNA may exist with or without PHI and both mRNA forms are found in the chicken gut and hypothalamus (Talbot et al., 1995). In contrast, the long form is found only in the turkey hypothalamus and comprises 4–6% of all VIP transcripts (You et al., 1995). The results of an in situ hybridization study reveal that PHI mRNA was expressed in very low abundance within the same hypothalamic areas expressing VIP mRNA (Chaiseha and El Halawani, 1999). There is no information about the physiological effect of PHI on PRL secretion in avian species.

The importance of VIP as a physiological PRL-releasing factor (PRF) in birds is very well established (for review see El Halawani et al., 1997). Passive immunization against VIP causes a reduction in plasma PRL and pituitary PRL mRNA and terminates egg incubation in chickens (Sharp et al., 1989; Talbot et al., 1991). Active immunization against VIP reduces plasma PRL levels and terminates incubation behavior in turkeys (El Halawani et al., 1995). To clarify whether an endogenous PHI is a genuine PRF in the domestic turkey, the present study was designed to compare the in vitro PRL response to VIP and PHI from primary pituitary cell culture, and to study the effect of PHI and VIP immunoneutralization on incubation behavior and PRL release.

2. Materials and methods

2.1. Experimental animals

Adult large white female Nicholas turkeys (10–13 kg) were used throughout the experiments. All birds were reared and housed in groups of eight in floor pens equipped with trap nests. Feed and water were constantly available. They were exposed to a photoperiod of 15L:9D at 50 lux of light. This was their second egg production period as adults. Trap nests were inspected from 8 AM until 4 PM for a total of six times per day. Birds were removed from the nest at each nest check and the accompanying eggs were removed at the same time. The number of times each bird entered the nest per day and the daily egg production were tabulated. If the birds nested continuously (i.e., were found in the nest six times per observation period) without laying eggs, they were considered to be incubating hens (Youngren et al., 1996). The animal protocols described in this paper were approved by the University of Minnesota Institutional Animal Care and Use Committee.

2.2. In vitro study

2.2.1. Pituitary cell dispersion

Dispersion of anterior pituitary cells from laying hens was obtained using the procedure previously described (Fehrer et al., 1985). The dispersed cells were incubated with medium M-199 (Life Technologies, Rockville, MD, USA) supplemented with 0.35 μg/ml $NaHCO_3$, 3% charcoal-stripped poults serum, 3% fetal calf serum (Sigma Chemical, St. Louis, MO, USA), 4.8 μg/ml Hepes (Sigma), 0.5% gentamycin sulfate (Sigma), 1% penicillin/streptomycin (Sigma), and 5 μg/ml amphotericin B (Sigma). Cell viability (90–95%) was determined using the trypan blue dye exclusion test and cell numbers were counted in a hemocytometer. The dispersed cells were initially incubated for 24 h in siliconized Erlenmeyer flasks at 38.5 °C in a 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% bovine serum albumin (Sigma). The culture medium was then replaced with fresh medium containing tVIP or tPHI or both at various doses (0, 10^{-12}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-4} M; 125,000 cells/treatment) for 3 h. Control cultures were replaced with fresh medium alone. At the end of each experiment, cells were pelleted and the media were collected and stored at −20 °C for determining PRL levels.

2.3. In vivo study

2.3.1. Active immunization with VIP and PHI and blood sampling

Birds were immunized 7 weeks before photostimulation with 125-μg synthetic tVIP or tPHI (El Halawani et al., 1995) conjugated to keyhole limpet hemocyanin (KLH, Peninsula Laboratories, Belmont, CA, USA). Control birds were injected with KLH mixed in Freund's complete adjuvant (Sigma). Booster immunizations (25 μg tVIP or tPHI in Freund's incomplete adjuvant) were administered 3 weeks prior to photostimulation and 4, 8, and 18 weeks following photostimulation.

Blood samples were collected from the brachial vein 7 and 1 weeks before photostimulation and on week 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 18, and 20 following photostimulation. Blood samples were heparinized and centrifuged at 2000*g* for 30 min and the plasma was stored at −20 °C until assayed for PRL and tested for the presence of antibodies for tVIP and tPHI.

2.3.2. VIP antibody titer

Plasma samples were prediluted (1:1000) in 0.5 M EDTA-phosphate buffered saline (PBS) and used as a source of primary antibody in a tVIP-binding assay (Mauro et al., 1992). ^{125}I-mono-iodinated tVIP was obtained through use of the Iodogen method and puri-

fied with reverse-phase high pressure liquid chromatography (Rozenboim et al., 1993).

2.3.3. PHI antibody titer

PHI antibody titer was determined by the enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtiter plates (ELISA plates, Costar, Cambridge, MA, USA) were coated with synthetic tPHI (0.2 μg in 100 μl carbonate buffer [pH 9.6] per well). Wells were washed three times with PBS containing 1% Tween (pH 7.4), and blocked with 200 μl of 1% bovine serum albumin (BSA, Sigma) per well for 2 h at room temperature. Test plasma samples were added at 1:1000 dilutions (the same dilution in the VIP-binding study) in PBS–Tween–1% BSA and incubated overnight at 4 °C. The wells were washed three times with PBS–Tween and second antibody (goat-anti turkey peroxidase conjugated, Kirkgard and Perry Laboratories, Gaithersburg, MA, USA) was added at a 1:1000 dilution in 100 μl of PBS–Tween–1% BSA. The wells were then incubated for 2 h at room temperature. After the wells were washed, enzyme substrate (100 μl of 0.1 phosphate–citrate buffer (PCB, pH 5.0) containing 10 mg of *o*-phenylenediamine dihydrochlororide (OPD, Sigma) and 10 μl of 30% H_2O_2/25 ml of PCB was added. The reaction was developed in the dark for 30 min and the degree of color change (optical density, OD) was measured in an ELISA plate reader at 492 nm. Results were corrected for nonspecific background by subtracting the OD from that of paired wells not coated with the adherence peptide but otherwise exposed to the identical procedure described above. Each plasma tested was determined in triplicate.

2.3.4. PRL radioimmunoassay

Plasma was assayed for PRL content using the homologous assay described by Proudman and Opel (1981). All samples from the same experiment were assayed simultaneously. All samples from each experiment were assayed in duplicate within a single assay.

2.3.5. Statistical analysis

The effects of tVIP and/or tPHI on in vitro PRL release were analyzed employing the General Linear Model procedure of the Statistical Analysis System (SAS, 1989). Each data point represents the mean ± SEM of three separate experiments, with three replicates per experiment. Significant differences in mean ± SEM among treatment groups were assessed using Tukey's Studentized Range Test at a significance level of $\alpha = 0.05$. All other data were analyzed with a repeated measure design. Multivariate comparisons of the time and treatment × time effects were made using MANOVA treats with the Wilks' Lambda statistic. Treatment comparisons of individual times were generated by contrast statements within the repeated measure program. Significant differences were reported when $P < 0.05$.

3. Results

3.1. Dose response effect of tVIP and tPHI on PRL release by anterior pituitary cells in vitro

tVIP increased PRL release by dispersed anterior pituitary cell cultures in a dose dependent manner with levels reaching maximum at 10^{-10} M (2.6 ± 0.2 μg/125,000 cells; Fig. 1). tPHI also increased PRL release in a dose dependent manner, with a maximum response at 10^{-8} M (2.2 ± 0.2 μg/125,000 cells). PRL response to tPHI decreased significantly ($P < 0.05$) at 10^{-6} M, the highest dose tested (from 2.6 ± 0.1 μg/125,000 cells at 10^{-7} M to 1.1 ± 0.1 μg/125,000 cells at 10^{-6} M).

3.2. Combined effects of tVIP and tPHI on PRL release by anterior pituitary cells in vitro

tPHI stimulated PRL secretion from anterior pituitary cell cultures in a dose dependent manner. However, at the highest dose of 10^{-4} M, tPHI inhibited PRL release to below basal level (Fig. 2; $P < 0.05$). The addition of 10^{-12} M tVIP to anterior pituitary cells with different tPHI concentrations did not alter ($P > 0.05$) the PRL response to tPHI. The same result was observed when 10^{-9} M tVIP was combined with different concentrations of tPHI. However, the 10^{-4} M dose of tPHI completely suppressed the tVIP-stimulated PRL secretion.

3.3. The effects of active immunization against tVIP and tPHI on plasma PRL level, nesting activity and egg production

3.3.1. Immunological response

In KLH–tVIP-immunized turkeys (Fig. 3A), mean VIP antibody titer level was undetectable before immunization.

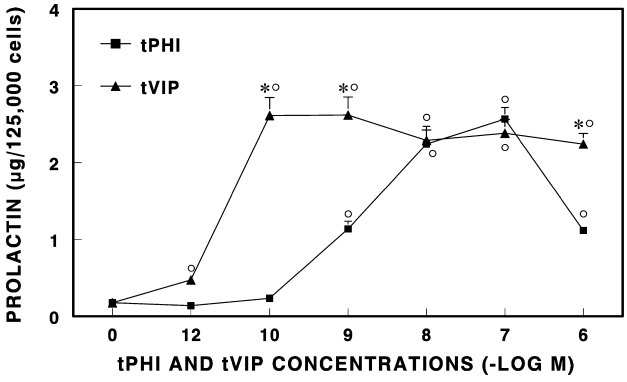

Fig. 1. PRL dose response to tPHI and tVIP treatments in primary anterior pituitary cell culture. Level of PRL present in 1.0 ml medium after dissociated turkey anterior pituitary cells were incubated for 3 h in the presence of various doses of tVIP and tPHI. Results are expressed as means ± SEM of three separate experiments, each with three replicates. *$P < 0.05$ compared to tPHI level; °$P < 0.05$ compared to zero level.

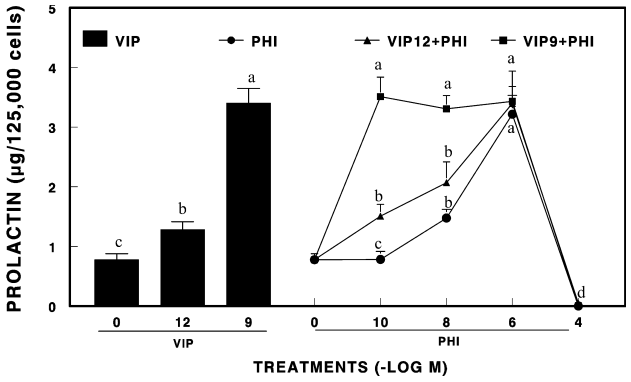

Fig. 2. The effects of tVIP and tPHI upon PRL release by anterior pituitary cell culture. Results are expressed as means;± SEM of three separate experiments, each with three replicates. Triangles and squares represent constant concentrations of VIP at 10^{-12} and 10^{-9} M, respectively, in the presence of various concentrations of PHI (10^{-10} to 10^{-4} M). Solid circles indicate PHI dose response from 0 to 10^{-4} M. See Fig. 1 for further details. Values with different letters are significantly ($P < 0.05$) different.

After the second immunization it increased to 16.0 ± 2.9% and peaked to a level of 31.9 ± 3.2% after the fourth immunization, following 9 weeks of photostimulation.

Peroxidase-mediated color reaction indicated the levels of antibodies to tPHI in plasma collected from KLH–tPHI-immunized turkeys (Fig. 3B). Before immunization and photostimulation the optical density (OD) was 0.076 ± 0.0003. The highest OD of 0.458 ± 0.045 was obtained after the second immunization, indicating maximum tPHI antibody titer, which then remained high until the end of the experiment.

3.3.2. Prolactin profile

Before photostimulation (day 0), PRL levels were the same in the control, tVIP immunized and tPHI immunized hens (Fig. 4A). Control and tPHI-immunized groups showed a significant ($P < 0.05$) increase in PRL levels with time following photostimulation. The PRL level of control and tPHI-immunized hens increased ($P < 0.05$) by week 6 of photostimulation. PRL level continued to increase until week 10 of photostimulation when peak levels were attained. After peaking, PRL levels declined ($P < 0.05$) at the end of the experiment. The tVIP-immunized group did not exhibit increased plasma PRL levels as the other groups; significantly lower PRL levels were maintained from week 6 of photostimulation until the end of the experiment. Overall, PRL levels for the entire experimental period were significantly ($P < 0.05$) lower in the tVIP-immunized turkeys as compared to either control or tPHI-immunized hens (Table 1).

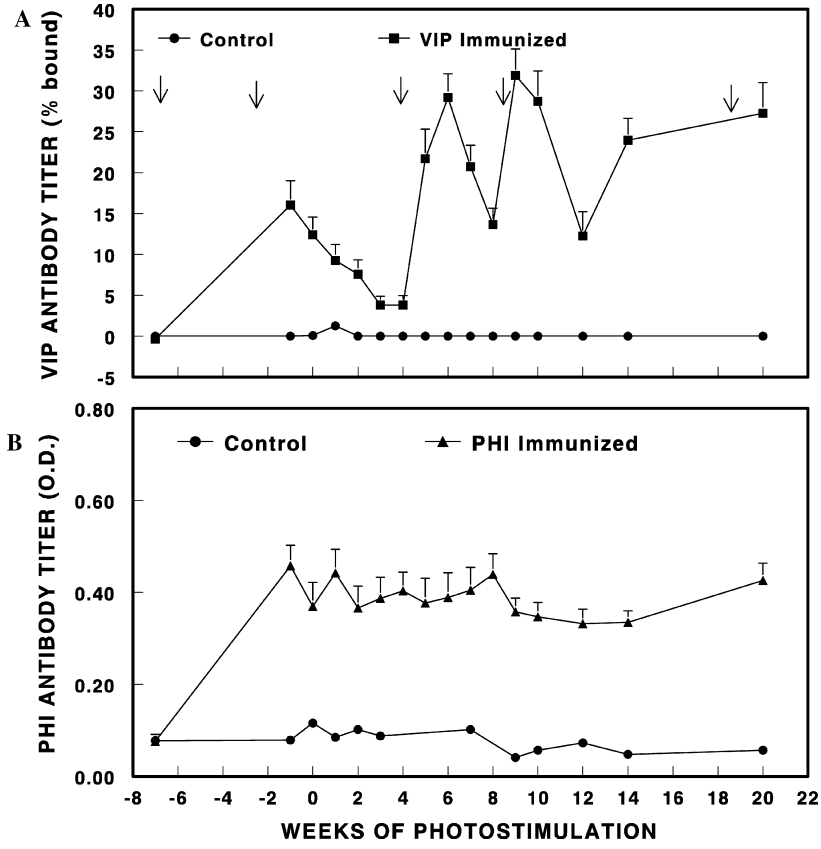

Fig. 3. (A) Plasma VIP antibody titer of turkey hens actively immunized with KLH–tVIP ($n = 23$) or KLH alone (control group; $n = 22$). (B) Plasma PHI antibody titer of turkey hens actively immunized with KLH–tPHI ($n = 14$) or KLH alone (control group; $n = 22$). Each point represents the mean and the vertical line represents the SEM. In some cases, the SEM is too small to be seen. Arrows indicate immunization schedule.

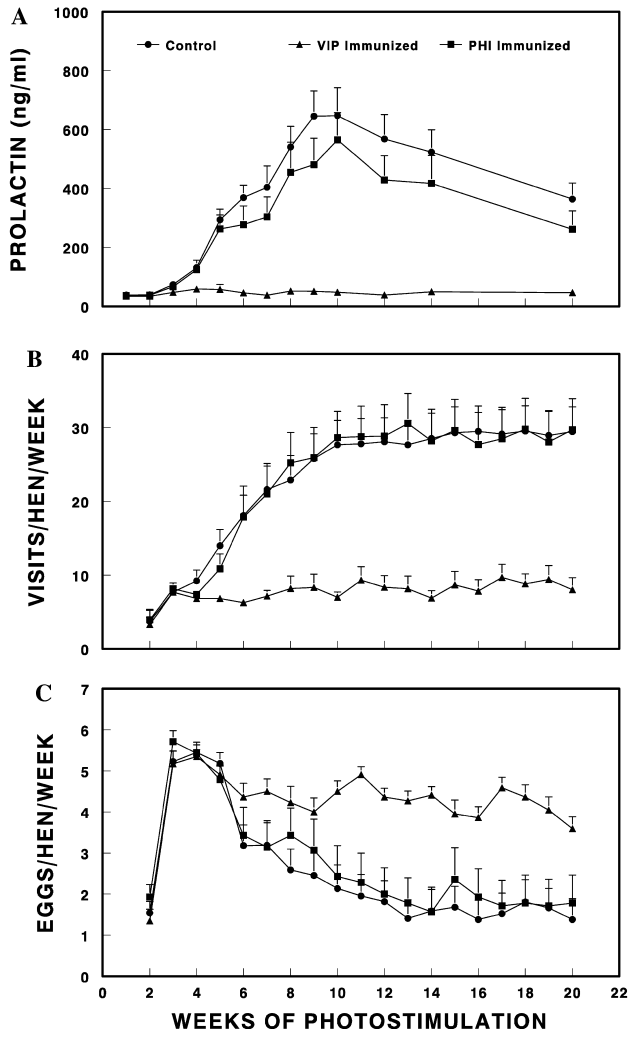

Fig. 4. Plasma PRL levels (A), nesting activity (B), and egg production (C) of turkey hens actively immunized with KLH alone (control, $n = 22$), tVIP–KLH ($n = 23$), and tPHI–KLH ($n = 14$) during a photo-induced reproductive cycle. Each point represents the mean and the vertical line represents the SEM.

3.3.3. Nesting activity

A continuous increase in nesting activity was observed from week 2 to week 9 of photostimulation in control and tPHI-immunized hens (Fig. 4B). From week 9 of photostimulation until the end of the experiment, nesting activity reached a plateau at a level of approximately 28 visits per hen per week. The tVIP-immunized group demonstrated significantly ($P < 0.005$) lower levels of nesting activity relative to the control and tPHI-immunized groups, from week 5 of photostimulation through the end of the observation period. Overall, for the entire experimental period, nesting activity in the tVIP-immunized group was significantly lower than that in the control and tPHI-immunized groups ($P < 0.05$; Table 1). The overall percentage of incubating hens (hens in each group that exhibit 30 or more nest visits per week) was significantly ($P < 0.05$) lower in the tVIP-immunized groups than the control and tPHI-immunized groups (Table 1). There were no significant differences ($P < 0.05$) in either nesting activity or in the percentage of incubating hens between KLH-immunized controls and KLH–tPHI-immunized turkeys (Table 1).

3.3.4. Egg production

There was no significant difference ($P > 0.05$) in egg production between the control and tPHI-immunized groups throughout the experiment (Fig. 4C; Table 1). Egg production peaked by week 3 of photostimulation (control from 1.5 ± 0.3 to 5.7 ± 0.2 eggs per hen per week; tPHI-immunized from 1.9 ± 0.3 to 5.7 ± 0.2 eggs per hen per week). Thereafter, egg production decreased gradually until the end of the experiment. The tVIP-immunized group exhibited a similar egg laying pattern to that of the control and tPHI-immunized groups in the first 8 weeks following photostimulation. However, from week 10 to the end of the experimental period, the tVIP-immunized group produced significantly ($P < 0.02$) more eggs than the other two groups. Overall egg production of the tVIP-immunized group was significantly higher ($P < 0.05$) than that of the control and tPHI-immunized groups (Table 1).

4. Discussion

The incubation of turkey anterior pituitary cells with either tVIP or tPHI-stimulated in vitro PRL secretion in a dose dependent manner. When female turkeys were actively immunized against tVIP or tPHI, the photo-induced rise in plasma PRL levels and the incidence of

Table 1
Reproductive characteristics of breeder turkey hens actively immunized against tVIP and tPHI during a 20-week reproductive period

Treatment	n	PRL (ng/ml)[a]	Nesting activity (visits per hen per week)[a]	Incubation (%)	Egg production (eggs per hen per week)[a]
Control (KLH-immunized)	22	307.7 ± 18.6^{A}	21.6 ± 2.4^{A}	72.7^{A}	2.5 ± 0.3^{A}
PHI-immunized	14	244.4 ± 19.4^{A}	21.6 ± 2.6^{A}	71.4^{A}	2.8 ± 0.5^{A}
VIP-immunized	23	44.0 ± 1.5^{B}	7.1 ± 0.9^{B}	13.0^{B}	4.2 ± 0.1^{B}

Visits per hen per week mean values were determined after calculating weekly egg production for each hen.
Values with different superscripts within the same column are significantly different ($P < 0.05$).
[a] Values represent group means (±SEM) based on averages from each hen over the 20-week photostimulation period.

incubation behavior were severely curtailed by tVIP, but not by tPHI immunoneutralization. These data accord with earlier results, that show VIP is the PRF in birds (El Halawani et al., 1997; Vleck and Patrick, 1999) and that PHI mRNA expression is very low in the turkey hypothalamus (Chaiseha and El Halawani, 1999; You et al., 1995). PHI, like VIP, is a PRF, but the lack of effect of PHI immunoneutralization on circulating PRL and its associated reproductive characteristics minimizes the physiological significance of PHI as a PRF in the turkey.

In mammals, both PHI and VIP are present in high concentrations in the hypothalamo-hypophyseal portal blood (Said and Porter, 1979; Shimatsu et al., 1983). VIP- and PHI-positive nerve fibers have been identified in the external layer of the median eminence (Ceccatelli et al., 1988, 1991; Hokfelt et al., 1982; Mikkelsen, 1989) and immunoreactive PHI concentration is twice that of VIP (Hokfelt et al., 1987). PHI and VIP are equally potent in stimulating PRL release in vivo and in vitro in the rat (Kaji et al., 1984; Ohta et al., 1985), and in humans, PHM acts as a physiological PRF as well (Sasaki et al., 1988). These results suggest that VIP and PHI are equally potent as PRFs in mammals. However, these findings are in sharp contrast to our findings. The results of the current study clearly demonstrate that tPHI is 100-fold less potent than tVIP in stimulating maximum PRL secretion in vitro. Also, co-administration of tVIP and tPHI does not exert an additive effect on PRL secretion in vitro.

Active immunization against tPHI or tVIP produced antibodies to their respective peptide. However, only birds immunized against tVIP had low plasma PRL levels and nesting activity, while egg production increased significantly. Porcine PHI had no effect on PRL secretion in ovariectomized turkey hens (Proudman and Opel, 1990). Kaji et al. (1984) suggested that in mammals VIP and PHI might affect PRL release through a synergistic action. Findings by Itoh et al. (1983) supported this possibility by demonstrating that VIP and PHI are created from the same precursor molecule and are stored within the same secretory granules, and may be simultaneously released into the hypophyseal portal blood and synergistically stimulate PRL release from the pituitary. However, our findings suggest that in the turkey, tVIP and tPHI have no synergistic effect and may act on the same receptors. Nevertheless, at the high dose of 10^{-4} M tPHI becomes an antagonist for PRL release, not only through inhibition of its own action but also through inhibition of tVIP action. Werner et al. (1983) suggested that the decreased effect of PHI at high concentrations might depend on mixed agonist/antagonist effects. The conclusion that VIP and PHI do not have a synergistic effect and that VIP is the main PRF in the turkey is further supported through the findings that the tPHI encoding exons encompass only 4–6% of the total tVIP transcript level in the turkey (You et al., 1995), and hypothalamic PHI mRNA expression shows no correlation with the state of prolactinemia (Chaiseha and El Halawani, 1999).

It is well established that immunization against VIP decreases PRL levels in the turkey and as a consequence nesting activity and the incidence of incubation behavior decrease significantly (El Halawani et al., 1996), while egg production increases (El Halawani et al., 1995, 1996). These are additional indicators for VIP as the physiological PRF. However, immunization against tPHI had no significant effect on PRL levels, nesting activity, incubation behavior, or egg production (this study). These findings cast doubt on PHI as a significant physiological PRF in the turkey, and further support VIP as the main turkey (avian) PRF.

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