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Stimulation of Prolactin Secretion from Turkey Anterior Pituitary Cells in Culture (42687)

J. A. PROUDMAN AND H. OPEL

Avian Physiology Laboratory, Agriculture Research Service, USDA, Beltsville, Maryland 20705

Abstract. Prolactin (PRL) secretion by monolayer cultures of turkey anterior pituitary cells was significantly increased (up to 44-fold) by vasoactive intestinal peptide (VIP), arginine vasotocin (AVT), and by an extract of turkey hypothalami (HE). Several other neuropeptides (including thyrotropin-releasing hormone) and neurotransmitters were ineffective in influencing PRL secretion at doses up to 10^{-6} *M*. The dynamic PRL response to HE and VIP was studied using superfused pituitary cells attached to microcarrier beads. HE, administered in 30-min pulses, resulted in a significant, dose-related increase in PRL secretion from a basal secretion rate of 2.32 ng/min/10^7 cells to a peak secretion rate of 127.13 ng/min/10^7 cells at the highest dose of HE tested (1 mg tissue-equivalent weight/ml). VIP significantly increased PRL secretion at all doses studied (from 10^{-10} to 10^{-6} *M*), with 10^{-8} *M* VIP producing a response similar to that observed with 1 mg/ml HE. A highly significant ($P < 0.001$) linear relationship was demonstrated between the log-dose of VIP administered and peak PRL secretion rate. These studies suggest that VIP, but not TRH, may be a physiological stimulus for PRL release in the turkey.

The control of prolactin (PRL) secretion in birds is unlike that of fish, amphibians, and mammals in that the avian hypothalamus exerts a stimulatory control over PRL secretion rather than an inhibitory control. Extracts of hypothalami from many avian species have been shown to stimulate PRL secretion by pituitaries cultured *in vitro* (1). However, the identity of the prolactin-releasing factor (PRF) in avian hypothalamic extract (HE) is uncertain. Neurotransmitters such as serotonin and norepinephrine have been shown to increase PRL secretion slightly through a direct effect on the pituitary, but have a greater effect when pituitaries are cocultured with whole hypothalami (1). Thyrotropin-releasing hormone (TRH), a potent stimulus for PRL secretion in mammals, increases PRL secretion from cultured chicken and pigeon pituitary cells (2), but has no consistent effect *in vivo* (1). None of these secretagogues provides as potent a stimulus to PRL secretion as HE.

Vasoactive intestinal peptide (VIP) functions as a PRF in the rat, and stimulates PRL secretion in man and monkeys (for review, see (3)). Our preliminary reports (4, 5) indicated that VIP stimulates PRL secretion in the turkey *in vitro* and *in vivo*. Others have shown a PRL response to VIP by the chicken (6) and the ring dove (7), and immunoreactive VIP has been localized immunohistochemically in the hypothalamus of the Japanese quail (8) and the chicken (6).

The present study was undertaken to screen several neurotransmitters and neuropeptides for PRF activity in turkey pituitary monolayer cultures, and to compare the dynamic response of superfused pituitary cells to HE and to VIP.

Materials and Methods. *Experiment I.* A monolayer culture system (9) was used to screen several neurotransmitters and neuropeptides for PRF activity. Treatments included turkey HE (50 to 800 μg tissue-equivalent weight/ml), TRH, dopamine, epinephrine, norepinephrine, arginine vasotocin (AVT), melatonin (each at log doses from 10^{-9} to 10^{-6} *M*), bombesin, and VIP (both at log doses from 10^{-10} to 10^{-6} *M*). An extract of brain cortex (CE) (200–800 μg tissue equivalent weight/ml) served as a treated control. Anterior pituitaries were obtained from 22 adult large white laying turkey hens. The glands were minced and the cells dispersed enzymatically using collagenase and pancreatin. The cells were cultured in monolayer flasks (25 cm^2) in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% MEM nonessential amino acids, 1 m*M* pyruvate, 25 m*M* Hepes buffer (pH 7.0), and antibiotics (Gentamycin, 50 μg/ml; amphotericin B, 0.5 μg/ml; penicillin G, 100

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U/ml; streptomycin, 100 μg/ml). Each flask received 1×10^6 cells in 5 ml of medium, and was incubated in a CO_2 incubator maintained at 37°C with an atmosphere of 95% air–5% CO_2. After 3 days, the medium was replaced with DMEM–5% FCS. On Day 5 of culture, the cells were rinsed with 2 ml of medium, and test substances were added in 5 ml of DMEM–5% FCS. After a 4-hr incubation, the medium was poured into tubes, refrigerated, and assayed within 24 hr for PRL using a homologous radioimmunoassay (RIA) (10). Intraassay variability was 2.4%. Cell counts of the established cultures were performed at the end of the experiment using nuclear staining (9). The mean (± SE) number of cells per flask was $3.40 \pm 0.16 \times 10^6$.

The HE and CE were prepared from hypothalami and brain cortex dissected from 12 of the pituitary donors. All tissues and extracts were maintained at 4°C throughout the extraction procedure. Tissue (110 mg wet wt of hypothalami) was homogenized in 0.1 *N* HCl in a ground glass homogenizer and centrifuged, and the supernatant was neutralized with NaOH. An additional precipitate was removed, and the extract was filtered through a 0.45-μm filter and lyophilized. The HE was dissolved in DMEM–5% FCS just prior to use, and was administered on a tissue equivalent-weight basis. The highest dose of HE used (800 μg/ml) represented 0.44 hypothalami per flask.

Experiment II. The dynamic response of cultured anterior pituitary cells to HE and to VIP was studied using a pituitary cell superfusion system (11). For each of two replicate experiments, anterior pituitary cells from seven small white laying turkey hens were dispersed enzymatically using collagenase (0.3%; 45 min) and trypsin (0.25%; 20 min). The cells were incubated for 30 min in calcium-free Hanks' solution containing 25 m*M* Hepes (pH 7.0) and 10 μg/ml DNase I. Dispersed cells were then suspended in a stirring flask (Techne, Inc., Princton, NJ) at a concentration of 5×10^5 cells/ml of DMEM–10% FCS. Total cell numbers cultured in the two replicate experiments were 6.9 and 5.7×10^7 cells, respectively. Approximately 0.5 g of Cytodex 3 microcarrier beads (Pharmacia Inc., Piscataway, NJ) was added to the culture flask and stirred (30 rpm) for 1 min every 2 hr for the first 24 hr of culture. At 24 hr, more than 90% of the cells were attached to the beads. DMEM was added to the culture to reduce the FCS concentration to 5%, and the culture was stirred constantly overnight. After about 40 hr in culture, the beads and their attached cells were allowed to settle briefly, and the media plus any unattached cells were removed by aspiration. The beads were rinsed once and resuspended in warm DMEM–5% FCS. Aliquots were pipetted into four sterile 0.7 × 4-cm disposable columns (Bio-Rad Laboratories, Richmond, CA) so that each contained about 1 ml of packed beads. Each column was connected to a four-channel peristaltic pump and the cells were superfused with DMEM–5% FCS at a flow rate of 200 μl/min. The effluent of each column was collected separately in a fraction collector at 10-min intervals. The volume of each fraction was recorded and the fractions were stored at −20°C until assay. All incubations were performed in a CO_2 incubator at 37°C in an atmosphere of 95% air–5% CO_2. In preliminary experiments, we found that a stable baseline level of PRL secretion was established within 90 min of the start of superfusion and was maintained for at least 24 hr. Superfused cells retained their ability to respond similarly to single or multiple challenges with HE for at least 30 hr. Therefore, collection of column fractions was started 90 min after superfusion began, and the first treatments were applied after an additional 90 min of superfusion.

In each of the replicate experiments, two superfusion columns were treated with CE (1000 μg tissue-equivalent weight/ml) and HE (250, 500, and 1000 μg/ml) prepared as in Experiment I. The other two columns were treated with VIP (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} *M*). Each treatment was applied for 30 min and followed by a 90-min recovery period. Treatment doses were applied in random order. At the end of the experiment the beads and cells were flushed from each column and incubated with trypan blue, and the number of cells was determined by counting liberated nuclei (9). PRL concentration was determined by RIA and the results expressed as nanograms of PRL secreted/minute/10^7 cells.

Reagents. DMEM, Hanks' solution, and MEM nonessential amino acids were purchased from K. C. Biologicals (Lenexa, KS). Gentamycin, pyruvate, DNase I, and Hepes came from Sigma Chemical Co. (St. Louis, MO). Collagenase (CLS) was purchased from Worthington (Bedford, MA). All other tissue culture reagents were obtained from GIBCO (Grand Island, NY). Synthetic VIP (human/porcine/rat) was supplied by Peninsula Laboratories (Belmont, CA), AVT by Bachem Fine Chemicals (Marina Del Ray, CA), and TRH by Beckman (Palo Alto, CA). All other secretagogues were obtained from Sigma.

Statistical analysis. Data from Experiment I were analyzed by analysis of variance. A Dunnett's procedure (12) was applied to compare treatment means with the control. The Dunnett's procedure was a two-sided comparison performed at the 0.01 level of significance to establish a significant rise or decline in PRL secretion. In Experiment II, an analysis of variance was conducted for each treatment. The sources of variation were replicates, columns within replicates and dosage of treatment. The latter was treated as a split-plot factor. A linear contrast was used to partition dosage effects to test for linear trends. Deviations from linearity (as measured on a log-dose scale) were also tested. All tests were performed at the 5% level of significance. A peak was defined as the highest PRL secretion rate observed during superfusion of a test substance, while the baseline for that peak was defined as the average of the two fractions collected immediately prior to treatment. Since treatments were applied to superfusion columns in random order, we have calculated the mean response profiles by reordering the secretion profiles of each column, aligning them at the start of each treatment period. For clarity, these profiles are presented in order of increasing dose of HE or VIP.

Results. *Experiment I.* Administration of HE, VIP, or AVT to monolayer cultures of turkey anterior pituitary cells resulted in a significant dose-related increase in PRL secretion during the 4-hr incubation (Fig. 1). PRL secretion of 5.93 ng/ml in control cultures rose sixfold after incubation with 200 μg/ml of a HE from laying turkey hens. PRL levels approximately doubled with each doubling of the dose of HE to 800 μg/ml, the highest dose tested. VIP at 10^{-10} *M* significantly increased PRL secretion to a level comparable to 200 μg/ml HE. A maximum (32-fold) PRL response to VIP was observed at a dose of 10^{-7} *M*. AVT increased PRL secretion at all doses tested and produced the greatest PRL response observed in this experiment—a 44-fold increase achieved with 10^{-6} *M* AVT. However, AVT was a less potent stimulus for PRL secretion than VIP at low doses. For instance, 10^{-9} *M* VIP produced a fourfold greater increase in PRL concentration than did 10^{-9} *M* AVT. At 10^{-7} *M*, the effectiveness of the two peptides was essentially equal. None of the other test substances (dopamine, epinephrine, norepinephrine, TRH, bombesin, serotonin, melatonin) elicited any significant rise or fall in PRL secretion at any of the doses tested. An extract of brain cortex produced no change in PRL secretion, and neither CE nor HE had any endogenous PRL activity as measured by RIA.

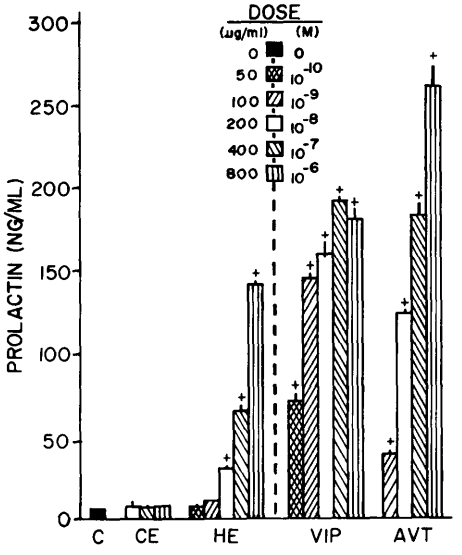

FIG. 1. Effect of hypothalamic extract (HE), vasoactive intestinal peptide (VIP), and arginine vasotocin (AVT) on prolactin (PRL) secretion by monolayer cultures of turkey anterior pituitary cells. Controls were either untreated (C) or received a cortical extract (CE). Treatment means (±SEM) significantly different ($P < 0.01$) from C are represented by +.

To preclude the possibility that the lack of PRL response to neurotransmitters may

have occurred because of oxidation of the secretagogues during incubation, we repeated the incubations with dopamine, epinephrine, norepinephrine, serotonin, and melatonin as described above, except that pituitary cells were obtained from medium white hens, and the media contained an antioxidant (10^{-4} *M* ascorbic acid). The results (not shown) confirmed our initial findings that these treatments produced no significant rise or fall in PRL secretion. VIP added to these cultures increased PRL secretion from 23.23 ng/ml in control cultures to 261.07 ng/ml at a dose of 10^{-7} *M*.

Experiment II. Superfusion of turkey anterior pituitary cells with HE resulted in a significant, dose-related increase in PRL secretion (Fig. 2). The highest dose of HE administered (1 mg/ml) caused a 55-fold increase in PRL secretion rate, from 2.32 to 127.13 ng/min/10^7 cells. CE at this dose caused a slight increase in PRL levels, but this change was not concurrent with the treatment period and was observed in only one of the four superfusion columns which received CE. The peak PRL response to log-dose increases in HE concentration showed a significant deviation from linearity, although linear contrast analysis indicated a significant linear trend, suggesting either that the dose range of HE used was too narrow to adequately define the dose–response relationship, or that the nature of the response changed with dose (possibly due to interaction of multiple secretagogues present in HE).

Application of 30-min pulses of VIP to superfused pituitary cells resulted in large, dose-related increases in PRL secretion rate (Fig. 3). The maximum PRL secretion rate observed in this experiment, 252.77 ng/min/10^7 cells, occurred in response to 10^{-6} *M* VIP. The peak PRL secretion rate increased linearly with increasing log-dose of VIP, as shown by a highly significant ($P < 0.001$) linear contrast and nonsignificant deviation from linearity. VIP administered at 10^{-8} *M* produced a PRL response similar to that seen with 1 mg/ml HE, both in terms of peak PRL secretion rate (154 vs 127.13 ng/min/10^7 cells, respectively) and total PRL secreted in the 2-hr treatment + recovery period (3.84 vs 3.53 μg/10^7 cells). Higher concentrations of VIP increased both the magnitude and the duration of the PRL response, such that 10^{-6} *M* VIP stimulated total PRL secretion of 10.03 μg/10^7 cells in 2 hr, and the PRL secretion rate remained elevated at about four times the basal secretion rate at the end of the 90-min recovery period.

Discussion. These studies have demonstrated the potent PRL-releasing effect of

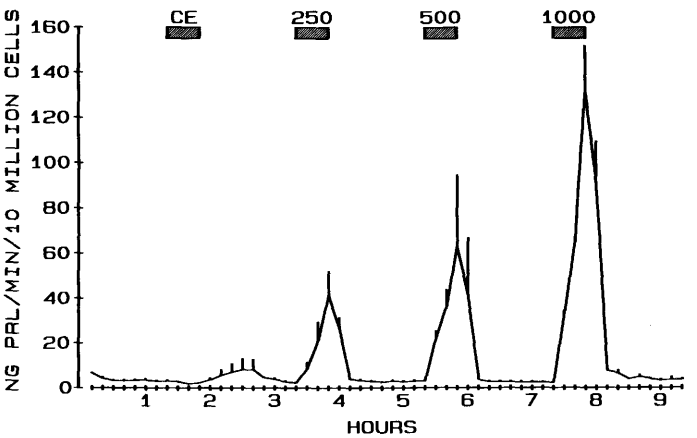

FIG. 2. Effect of HE (doses indicated in μg tissue-equivalent weight/ml) or CE (1000 μg/ml) on PRL secretion by superfused turkey anterior pituitary cells. Treatments were applied for 30 min (shaded bar) at 200 μl/min and followed by a 90-min recovery period. Each point represents mean ± SEM of four replicate columns. Each column received treatments in random order, but data are arranged in order of ascending dose of HE.

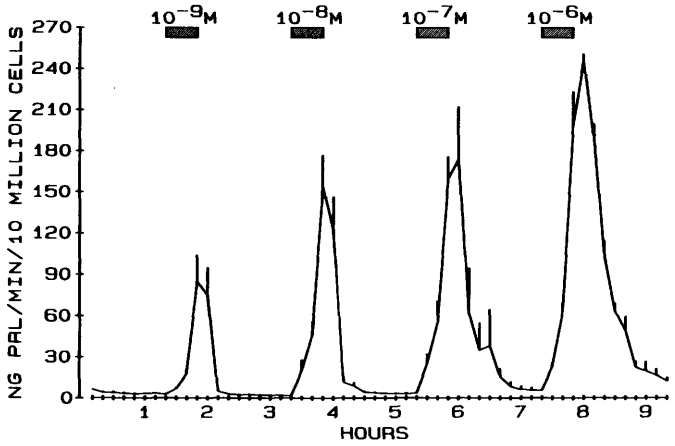

FIG. 3. Effect of VIP on PRL secretion by superfused turkey anterior pituitary cells. Treatments were applied for 30 min (shaded bar) at 200 μl/min and followed by a 90-min recovery period. Each point represents mean ± SEM of four replicate columns. Each column received treatments in random order, but data are arranged in order of ascending dose of VIP.

turkey HE and mammalian VIP on cultured turkey pituitary cells. A significant (6-fold) increase in PRL concentration was observed in monolayer cultures receiving 0.11 tissue equivalents of HE, while a larger (12-fold) increase occurred in response to the lowest dose of VIP tested (10^{-10} *M*). Superfused turkey pituitary cells secreted PRL at a low basal level and responded in a dynamic, dose-related manner to both HE and VIP. This response was highly reproducible between separate superfusion columns and between different pituitary cells preparations. The basal PRL secretion rate was much lower than that found with superfused rat cells (13) due to the species difference in hypothalamic control of PRL secretion (stimulatory vs inhibitory), yet both species respond strongly to physiological doses of VIP. VIP has been reported to stimulate PRL release by static cultures of rat pituitary cells (14, 15), and by superfused rat (13), and monkey (16) pituitary cells. The lowest doses of VIP effective in our studies are comparable to the levels of VIP (10^{-9} to 10^{-10} *M*) found in the hypophyseal portal blood of the rat (17). VIP likely functions as an authentic PRF in the rat, since it is found in both the hypothalamus and the pituitary, is present in much higher concentration in hypophyseal portal blood than in the peripheral circulation, and is able to stimulate PRL secretion *in vivo* and *in vitro* (3). Antiserum to VIP abolishes the normal *in vivo* PRL response to neurogenic stimuli (18). Similar evidence of a PRF role for VIP in avian species is incomplete. The present *in vitro* studies and our *in vivo* experiments (19) provide the first evidence for such a role in the turkey. VIP-immunoreactive nerve fibers have been identified in the median eminence of the Japanese quail, in close contact with portal capillaries (8). VIP-immunoreactive cell bodies have been located in the basal hypothalamus of the chicken, with fibers terminating in the external layer of the median eminence (6). VIP has been reported to cause a small increase in PRL secretion by cultured chicken hemipituitaries (20), and by whole pituitaries from nest-deprived incubating Bantam chickens (6). Pituitaries from laying Bantam hens, however, do not respond to 10^{-7} *M* VIP, a dose which elicited the maximum response of our monolayer cultures of pituitary cells from laying turkeys. The reason for this difference is not clear, but examples of substantial differences in the apparent secretion of PRL by chickens and turkeys are abundant in the literature. Studies directly measuring VIP levels in the brain, pituitary, or portal circulation of any bird are lacking.

AVT also caused large increases in PRL secretion in cultured turkey pituitary cells, although it was less effective at low doses

than was VIP. However, in studies using unrestrained, ovariectomized adult turkey hens (19), we have found that iv administration of AVT at doses ranging from 0.01 to 1 μg/kg of body wt caused no change in PRL levels (n = 3–7/dose), although the highest dose caused immediate convulsions and death in four of seven hens. We, therefore, consider it unlikely that AVT could function as a physiological PRF in the turkey.

Although we have not conducted detailed studies of the specificity of the PRL response to VIP and AVT *in vitro,* we have observed (4) that monolayer cultures secrete growth hormone (GH) in response to VIP but not AVT. The maximal GH response occurred with 10^{-6} M VIP, a dose 100 times that required for TRH to produce a similar GH response. Macnamee *et al.* (6) have reported a small increase in circulating luteinizing hormone concentration but no change in GH concentration in incubating Bantam chickens receiving VIP *in vivo.*

TRH is highly effective in stimulating PRL secretion in mammals, and direct comparison with VIP in the rat superfusion system has shown TRH to be more effective on a molar basis than VIP in causing PRL release (13). TRH and VIP are equally effective in stimulating PRL secretion from monkey pituitary cells (16). This is clearly not the case in the turkey, since TRH was without effect on PRL secretion by monolayer cultures at dose levels up to 10^{-6} M. By contrast, 10^{-8} M TRH produced a nearly 10-fold increase in GH secretion by these same cultures (4). Fehrer *et al.* (21) also found no effect of TRH on prolactin secretion by turkey pituitary cells. These results differ somewhat from those of chicken studies, which have generally found TRH to have a small stimulatory effect on *in vitro* PRL secretion (for review, see (1)). The reason for this discrepancy is unclear, but it seems likely that any PRL-releasing activity of TRH is minor compared to the consistently high PRF activity found in turkey HE, and to the potency of TRH in mammals.

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