PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 187, 455–460 (1988)

Stimulation of Prolactin Release in Turkeys by Vasoactive Intestinal Peptide (42688)

H. OPEL AND J. A. PROUDMAN

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

Abstract. Vasoactive intestinal peptide (VIP) is a potent releasor of prolactin in birds. The main purpose of this study was to identify its site of action. Synthetic porcine VIP administered intraatrially to freely moving ovariectomized (OVX) turkeys induced an elevation of circulating PRL within 15 min in a dose-related manner. Removal of hypothalamic control of PRL release by surgical disconnection of the neurohemal regions of the median eminence did not significantly diminish the PRL response to VIP. Intraatrial injection of eledoisin or bradykinin into OVX hens did not influence PRL secretion, indicating that the PRL releasing activity of VIP is probably not attributable to its vasodilatory action. These results support the possibility that VIP is an authentic prolactin releasing factor (PRF) in birds.

Vasoactive intestinal peptide (VIP) is an octacosapeptide whose sequence differs in the pig and chicken in amino acid positions 11, 13, 26, and 28 (1, 2). Much evidence indicates that VIP is a prolactin releasing factor (PRF) in mammals (3, 4). Limited evidence suggests that VIP is a possible candidate for PRF in birds. Numerous VIP-immunoreactive neurons in the avian hypothalamus project axons to the external layer of the median eminence, suggesting that VIP plays a role in control of pituitary function (5, 6). Porcine VIP has been shown to stimulate secretion of PRL from cultures of anterior pituitary cells of the turkey (7) and chicken (8) and to evoke *in vivo* release of PRL in broody chickens (6) and in nonbreeding and brooding ring doves (9). The available data from birds do not provide direct information on the endogenous site of action of VIP in stimulating PRL secretion.

This study assesses PRL releasing effects of VIP in the ovariectomized turkey and whether such effects are exerted centrally or directly on the pituitary. Commercially available fragments of VIP are tested to examine the possibility that PRL releasing activity, like other hormonal activity (10), requires the whole VIP molecule. In addition, the activities of the potent hypotensive agents eledoisin and bradykinin (11) are measured to determine if the PRL releasing activity of VIP might be attributable to its potent vasodilatory action on extracranial and cerebral blood vessels (12). Finally, the activities of arginine vasotocin (AVT), shown to have *in vitro* PRL releasing activity in the turkey (7), and angiotensin II, shown to release PRL in the female rat (13), are compared to the PRL releasing activity of VIP.

Materials and Methods. *Animals.* Mature ovariectomized (OVX) Nicholas large white turkeys (6–8 kg) were caged individually under a 14:10 light:dark cycle (lights on at 0300 hr) with free access to feed and water. All turkeys used in this study were OVX at least 2 months before experimentation. OVX turkeys were chosen to avoid changes in plasma PRL associated with ovulation.

Cannulation. Four days before initiation of the experiments the OVX hens were anesthetized with pentobarbital (20 mg/kg iv, supplemented as needed) and fitted with indwelling right-atrial cannula by a method previously described (14). On completion of surgery hens were caged individually in a single-tiered cage unit (capacity 12 hens) bolted to one wall of an environmentally controlled room (lights on 0300–1700 hr, 23 ± 1°C, low noise level). Each hen was tethered by a back harness and flexible stainless steel tether to allow freedom of movement throughout the cage. The cannula was extended through the tether to a swivel mounted on top of the cage and then passed from the swivel through the wall holding the cage into an adjoining room where test solutions could be injected and blood samples withdrawn without disturbing the hens. The

0037-9727/88 $1.50

environmentally controlled room was not entered on the day before and during the 2 days of blood sampling.

Peptide injection and blood sampling. Synthetic porcine VIP, porcine VIP fragment 10-28, chicken VIP fragment 16-28, angiotensin II, bradykinin (Sigma Chemical Co., St. Louis, MO), and eledoisin (Peninsula Laboratory, Belmont, CA) were dissolved alone in 0.9% saline immediately before injection in a volume of 1 ml over a period of 10 sec.

An identical 2-day injection and blood sampling protocol, in which each hen served as its own control, was used to test PRL releasing action of each peptide. On Day 1, a preinjection blood sample (2.5 ml) was drawn from groups of 10–12 hens, the saline vehicle was injected over a period of 10 sec and additional samples were collected at 5, 10, 15, 30, 60, and 120 min following injection. After each collection, the cannula was flushed with 1.5 ml of heparinized saline (10 U/ml). On Day 2, hens were randomly selected to receive one of several doses of the test peptide, and blood samples were collected according to the Day 1 procedure. This protocol was repeated until each treatment had been administered to six hens. No hen was used more than once experimentally. In a final experiment examining the site of action of VIP, the same procedure was followed, except that a high dose of VIP was tested in seven median eminence (ME) deafferented OVX hens and four sham-deafferented controls.

A preliminary study of 12 OVX hens given 7.9 μg/kg body wt of VIP or 1 ml of saline on Day 1 and the alternate injection on Day 2 had established that the order of injection of peptide and vehicle had no effect on the PRL response to VIP. However, if a hen had sensed that blood was being withdrawn on either day, plasma PRL levels rose significantly. The source of the sensation appeared to be inadvertant contact between the flexible tip of the cannula and the wall of the atrium. The response was abrupt cessation of the behavior in progress followed by elevation of the head to an alert position. Therefore, to minimize procedural-induced changes in PRL, closed circuit television was used to monitor the behavior of each hen during each blood collection and all samples from any hen showing any indication that she could detect withdrawal of a sample were excluded from the analysis of results. Plasma samples used in the analyses were stored frozen at −70°C until assayed.

Radioimmunoassay and statistical analysis. Plasma levels of PRL were measured by homologous radioimmunoassay (15). Interassay variability was 12.5% and intraassay variability was 8.0%. Potency estimates were calculated using the spline-function program of the LKB Model 1270 gamma counter.

Analysis of variance was used to establish differences over treatment and differences over time. Where treatment by time interactions were present, Dunnett's procedure (16) was used to compare means from a given sampling time to the zero time. The Dunnett's procedure used a two-sided comparison at the 0.05 level of probability to establish a significant rise or decline in PRL levels.

Deafferentation. In birds, the hypophyseal portal vessels do not form part of the infundibular stalk as they do in mammals (17). Therefore, rather than attempt stalk section to completely deprive the anterior pituitary of its portal circulation from the hypothalamus, we transected the base of the hypothalamus at a level about 1.0 mm above the neurohemal regions of the anterior and posterior divisions of the ME which contain all point to point connections with the portal vessels. In addition to the ME, the island of neural tissue below the cut (Fig. 1) included approximately the lower one-fourth of the basal infundibular nucleus, as defined by Oksche *et al.* (18) and the entire posterior lobe of the pituitary. Deafferentation (transection) of the ME of chronically cannulated OVX hens was performed 2 days before challenge with VIP. The microsurgical knife assembly used consisted of a ventricle guide tube made from 20 gauge stainless steel tubing and an L-shaped knife (angle 90°; radius 3.2 mm) made from 24 gauge piano wire. Flat edges were ground into opposite sides of the wire and the resultant double-edged blade was carefully sharpened. The hen was fixed in the frame of a stereotaxic instrument so that the top of the skull was horizontal (height of frontal and parietal bones equal). The knife, with blade pointed posteriorly, was lowered

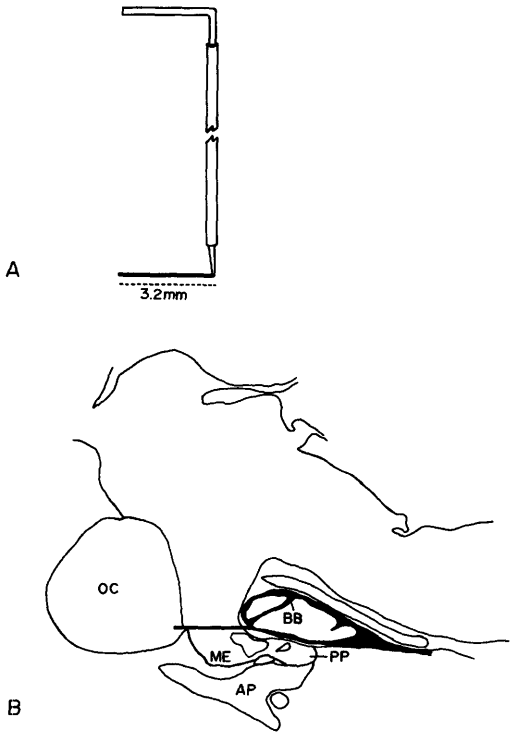

FIG. 1 (A) Diagram of stereotaxic knife. (B) Sagittal section of hypothalamus showing position of cut (thick horizontal line) used to isolate neurohemal region of median eminence and thus prevent hypophyseal portal blood flow to anterior pituitary. Abbreviations: AP, anterior pituitary; BB, basisphenoid bone; ME, median eminence; OC, optic chiasma; PP, posterior pituitary.

through the midsagittal sinus according to predetermined stereotaxic coordinates so that the tip came to rest on the posterior rim of the entrance to the sella turcica formed by the dorsal surface of the basisphenoid bone. The knife was then moved 0.5 mm anteriorly to clear the rim of bone, lowered vertically 1.5 mm into the sella, and moved posteriorly under the rim until the tip pressed against the periosteum of the posterior dorsal wall of the sella (Fig. 1). At this position the knife blade was rotated 180° to the left and right 6–8 times, rotated 360° twice, and then was returned to the midline and withdrawn from the brain. For sham operation, the knife was lowered through the midsagittal sinus to touch the rim of the basisphenoid bone and then withdrawn.

Water intake measurements. Since eledoisin and angiotensin II are known to have dipsogenic action in birds (11), water intake was measured during tests of these peptides. A precalibrated water bottle with tip immersed in a drinking cup was attached to each cage immediately before the test injection and left in place until the last blood sample was drawn. The difference in milliliters between the beginning and ending water volume in each bottle was measured as the water intake over 2 hr.

Histology. Formalin fixed frozen brains were cut into 25-μm sections and stained with luxol blue-cresyl violet. Deafferented hens were used in this study only if examination of serial sections showed that the neurohemal ME and pituitary stalk were completely transected and that no recognizable clusters of neuronal cell bodies were present in the portion of the basal tuberal nucleus below the knife cut.

Results. Figure 2 shows that single intraatrial injections of 3.9 μg/kg body wt of VIP produced two- to threefold increases ($P < 0.05$) in plasma PRL levels in freely moving OVX turkeys, while injections of 7.9 or 15.8 μg/kg body wt produced four- to sixfold increases ($P < 0.05$). Injections of 0.78 μg/kg of VIP or injections of the saline vehicle had no effect. Irrespective of dose, injections of VIP produced peak PRL levels within 15 min of injection which were followed by declines to pretreatment levels within 120 min.

Injections of porcine VIP fragment 10-28 (5, 10 μg/kg body wt), chicken VIP fragment 16-28 (5, 10 μg/kg), AVT (0.01, 0.1, 0.5, 1.0 μg/kg), Angiotensin II (10, 20, 40 μg/kg), eledoisin (5, 50, 100 μg/kg), or bradykinin (4, 16, 32 μg/kg) had no significant effect on PRL release (data not shown). The two highest doses of AVT caused panting and muscular incoordination in all hens and led to convulsions and death within 3 min in some hens. Injections of angiotensin II and eledoisin led to large dose-dependent increases in water consumption. Vigorous but completely normal drinking began within 2–3 min after injection.

Plasma PRL profiles of seven ME-deafferented and four sham-deafferented OVX hens given intraatrial injections of 10 μg/kg body wt VIP are presented in Fig. 3. VIP evoked a 3- to 15-fold rise ($P < 0.05$) in PRL levels in deafferented hens and a 4- to 8-fold rise (P

< 0.05) in sham-operated hens. The difference in the magnitude of the response between the two treatment groups was not statistically significant. Figure 3 indicates that maximum PRL levels were reached at 5 min after VIP injection in deafferented hens and at 15 min after injection in sham-operated hens. However, this apparent difference is accounted for by a 15-fold rise (peak of 150.29 ng/ml) by 5 min in one deafferented hen. Peak values were reached at 15 min after VIP treatment in the remaining six hens (3- to 8-fold rise, mean PRL value 39.64 $\pm$ 8.25 ng/ml). Based on the data from the six hens, the magnitude and duration of the PRL response evoked by administration of 10 μg/kg body wt VIP to deafferented and sham-deafferented OVX hens are similar to those obtained in the first experiment by administration of 7.8 or 15.6 μg/kg body wt of VIP to OVX hens not subjected to cranial surgery.

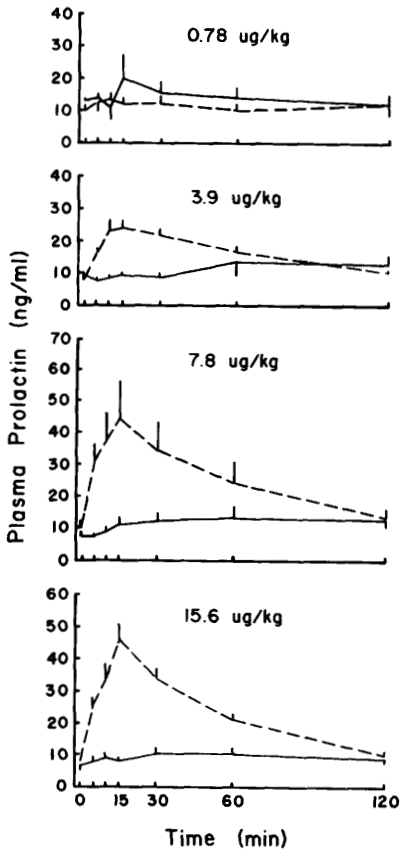

FIG. 2. Effects of a single intraatrial injection of saline or 0.78, 3.9, 7.8, or 15.6 μg/kg body wt of VIP on plasma prolactin levels in unrestrained OVX turkeys. The injections were given immediately after collection of a pretreatment sample at Time 0. Solid line shows effect of injection of saline on Day 1. Broken line shows effects of injections of VIP into the same turkeys on Day 2. Each point represents mean $\pm$ SEM (vertical bar) of six turkeys.

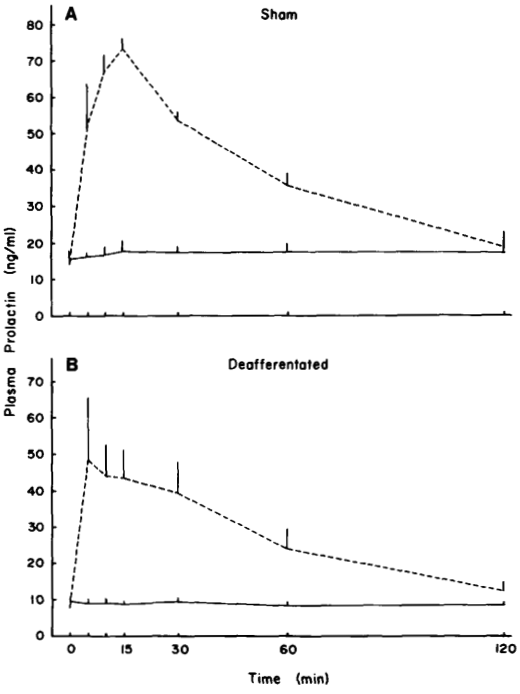

FIG. 3. Effects of VIP on PRL secretion in unrestrained ME-deafferented OVX turkeys (A) Sham-operated controls ($n = 4$); (B) deafferented ($n = 8$). All birds were tested on Days 1 and 2 after cranial surgery. See legend to Fig. 2 for further details.

Mean pretreatment levels of PRL in all seven samples taken on Sampling Day 1 and in the single (0 min) sample taken on Day 2 were consistently 40–50% lower in deafferented than in sham-deafferented hens. There was no indication, in either treatment group, of a progressive decline in plasma PRL with time after cranial surgery. Other than the lower basal levels of PRL, the only apparent biological effects of deafferentation were severe polyuria and polydypsia present at the onset of the light period on the day after surgery. The latter effects are an obvious conse-

quence of diabetes insipidus produced by transection of the posterior pituitary stalk.

Discussion. VIP was a potent stimulus for PRL release in the OVX turkey. The demonstration that the PRL releasing action of VIP was not significantly diminished by surgical isolation of the neurohemal ME and anterior pituitary from hypothalamic influences indicates that the peptide exerts its effects, at least in part, by direct action on the pituitary. This conclusion receives additional support from our earlier study (7) in turkeys showing VIP is highly potent in stimulating secretion of PRL from pituitary cells of laying hens cultured *in vitro.* The failure of the hypotensive agents eledoisin and bradykinin to induce PRL release *in vivo,* as well as the effectiveness of VIP *in vitro,* argues against the possibility that the PRL releasing activity of VIP is attributable to its hypotensive properties.

The findings that synthetic porcine VIP fragment 10-28 and synthetic chicken VIP fragment 16-28 did not stimulate PRL secretion were not unexpected. Studies of biological activities of synthetic porcine VIP and VIP fragments in mammalian assay systems not based on PRL release, such as *in vivo* and *in vitro* systems measuring vasodilation (10, 12) and *in vivo* systems measuring smooth muscle relaxation (10), show that only the whole molecule or fragment 2-28 exhibits activity close to natural porcine VIP.

The observation that basal levels of plasma PRL were lowered rather than elevated by transection of the ME is consistent with the hypothesis that the major hypothalamic influence on PRL secretion in birds is stimulatory. From this one might expect that deafferentation of the ME would lead to at least partial necrosis of the anterior pituitary and some loss of secretory function among lactotrophs. We did not examine possible short-term necrotic changes in the anterior pituitaries of our deafferented hens because we lacked the appropriate immunocytochemical techniques. An early histological study in the Pekin duck (19) shows that lactotrophs have lost secretory function by 1 month after permanent transection of the portal veins.

Our observations on PRL releasing potency of VIP in OVX turkeys are consonant with reports in bantam chickens (6) and ring doves (9) showing that systemic injection of VIP evokes a fivefold increase in plasma PRL concentrations within 10 min. Our earlier *in vitro* study demonstrating high potency of VIP in stimulating PRL release from cultures of turkey pituitary cells (7) contrasts with an *in vitro* study in the chicken (8) indicating relatively low potency of VIP in evoking PRL secretion from cultures of hemipituitaries. We found no evidence that angiotensin II, a candidate for PRF in the rat (13), evoked PRL release in the turkey. Although AVT appears to be a potent releaser of PRL in cultured turkey anterior pituitary cells (7) this peptide did not release PRL *in vivo.*

Another peptide that has an uncertain role as an important mediator of PRL secretion in birds is TRH (see Ref. (20) for review). This peptide stimulates PRL release from pigeon (21) and chicken (8, 20, 21) pituitaries *in vitro,* but does not stimulate PRL release in immature chickens (21). Fehrer *et al.* (22) reported that TRH induces PRL release in the turkey *in vivo,* but not *in vitro.* Evidence from this laboratory indicates that TRH has neither *in vivo* or *in vitro* PRL releasing action in turkeys (7, 23).

In conclusion, the results of this study and our earlier *in vitro* study (7) strongly support the possibility that VIP is a PRF in turkeys. In addition to this evidence, it is important to know whether VIP is present in hypophyseal portal blood and whether immunoneutralization of endogenous VIP modifies well-defined changes in PRL secretion associated with important physiological events, such as the dramatic rise in circulating PRL at the onset of incubation behavior.

The authors thank Dr. Estelle Russek, Department of Animal Sciences, University of Maryland, for performing the statistical analyses and Beverly Russel and Gaynelle Campbell for technical support.

1. Said SI, Mutt V. Isolation from porcine intestinal wall of a vasoactive octacosapeptide related to secretion and to glucagon. Eur J Biochem **28**:199–204, 1972.
2. Nilsson A. Structure of the vasoactive intestinal octacosapeptide from chicken intestine. The amino acid sequence. FEBS Lett **60**:322–326, 1975.
3. Nicosia S, Oliva D, Giannattasio G, Spada A. The role of vasoactive intestinal polypeptide (VIP) as a

hypothalamic neurohormone. J Endocrinol Invest **6**:235–242, 1983.
4. Abe H, Engler D, Motlitch ME, Bollinger-Gruber J, Reichlin, S. Vasoactive intestinal peptide is a physiological mediator or prolactin release in the rat. Endocrinology **116**:1383–1390, 1985.
5. Mikami S-I, Yamada S. Immunohistochemistry of the hypothalamic neuropeptides and anterior pituitary cells in the Japanese quail. J Exp Zool **232**:401–417, 1984.
6. Sharp PJ, Macnamee MC, Talbot RT, Sterling RF, Hall TR. Aspects of neuroendocrine control of ovulation and broodiness in the domestic hen. J Exp Zool **232**:475–483, 1984.
7. Proudman J, Opel H. Stimulation of prolactin secretion from turkey anterior pituitary cells. Proc Soc Exp Biol Med **187**: 448–454, 1988.
8. Hall TR, Chadwick A. Peptide-stimulated release of prolactin from the fowl anterior pituitary. Experientia **41**:196–198, 1985.
9. Lea RW, Vowles DM. Vasoactive intestinal polypeptide stimulates prolactin release *in vivo* in the ring dove (*Streptopelia risoria*). Experientia **42**:420–422, 1986.
10. Bodansky M, Klausner YS, Said SI. Biological activities of synthetic peptides corresponding to fragments of and to the entire sequence of the vasoactive intestinal peptide. Proc Natl Acad Sci USA **70**:382–384, 1973.
11. De Caro G, Mariotti M, Massi M, Micossi LG. Dipsogenic effect of angiotensin II, bombesin, and tachykinins in the duck. Pharmacol Biochem Behav **123**:229–233, 1980.
12. Fournier A, Saunders JK, St-Pierre S. Synthesis, conformational studies and biological activities of VIP and related fragments. Peptides **5**:169–177, 1984.
13. Steel MK, Negro-Vilar A, McCann SM. Effect of angiotensin II on *in vivo* and *in vitro* release of anterior pituitary hormones in the female rat. Endocrinology **109**:893–899, 1981.
14. Opel H, Proudman JA. Two methods for serial blood sampling from unrestrained, undisturbed turkeys with notes on the effects of acute stressors on plasma levels of prolactin. Poult Sci **63**:1644–1652, 1984.
15. Proudman JA, Opel H. Turkey Prolactin: Validation of a radioimmunoassay and measurement of changes associated with broodiness. Biol Reprod **25**:375–384, 1981.
16. Steel RDG, Torrie JH, Ed. Principles and Procedures of Statistics. New York, McGraw-Hill, 1960.
17. Assenmacher I. Reproductive Endocrinology: The hypothalamohypophysical axis. In: Farner DS. Ed. Breeding Biology of Birds. Washington, DC, National Academy of Sciences, pp158–191, 1973.
18. Oksche A, Zimmermann P, Oehnke H-J. Morphometric studies of tubero-eminental systems controlling reproductive functions. In: Knigge KM, Scott DE, Weindl A, Eds. Brain Endocrine Interaction Median Eminence: Structure and Function. Basel, Kruger, pp142–153, 1971.
19. Assenmacher I, Tixier-Vidal A. Repercussions de la section des veines portes hypophysaires sur la prehypophyse du canard pekin male, entier ou castre. Arch Anat Micros Morphol Exp **53**:83–108, 1964.
20. Harvey S, Scanes CG, Chadwick A, Bolton NJ. The effect of thyrotrophin-releasing hormone (TRH) and somatostatin (GHRIH) on growth hormone and prolactin secretion *in vitro* and *in vivo* in the domestic fowl (Gallus domesticus). Neuroendocrinology **26**:249–260, 1978.
21. Hall TR, Chadwick A, Bolton NJ, Scanes CG. Prolactin release *in vitro* and *in vivo* in the pigeon and the domestic fowl following administration of synthetic thyrotrophin-releasing factor (TRF). Gen Comp Endocrinol **25**:298–306, 1975.
22. Fehrer SC, Silsby JL, Behnke EJ, El Halawani ME. The influence of thyrotrophin releasing hormone on *in vivo* prolactin release and *in vitro* prolactin, luteinizing hormone, and growth hormone release from dispersed pituitary cells of the young turkey (*Meleagris gallopavo*). Gen Comp Endocrinol **59**:64–72, 1985.
23. Proudman JA. Growth hormone and prolactin response to thyrotrophin releasing hormone and growth hormone releasing factor in the immature turkey. Proc Soc Exp Biol Med **175**:79–83, 1984.

Received November 11, 1987. P.S.E.B.M. 1988, Vol. 187.
Accepted December 7, 1987.