A Vasoactive Intestinal Peptide Binding Component in Hen Granulosa Cells (43912)

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Abstract. **In radioligand assays, the vasoactive intestinal peptide (VIP) binding component in the membrane fraction of granulosa cells of the ovary of the hen was shown to possess characteristic properties of a receptor, such as reversible binding, binding specificity, high-affinity, and limited capacity. The binding site was of a single class. The binding affinity was higher in the largest (F_1) and the second largest follicle (F_2) than in the third largest follicle (F_3), and the binding capacity was greater in F_2 and F_3 than in F_1. During the ovulatory cycle, changes in affinity and capacity were observed only in F_1 shortly before ovulation. The results suggest the presence of VIP receptor in the hen granulosa cells and its binding is assumed to be related to the follicular growth and ovulation.** [P.S.E.B.M. 1995, Vol 209]

Vasoactive intestinal peptide (VIP), first isolated from porcine small intestine, is composed of 28 amino acid residues (1). Chicken VIP (cVIP) is different from mammalian VIP in its amino acid sequence at position 11, 13, 26, and 28, although the number of amino acid residues is the same (2). Various VIP-mediated actions are known in the ovary of mammals, such as induction of cAMP formation (3), enhancement of activity of aromatase (3) and plasminogen activator (4), stimulation of steroid hormone production in theca (5) and granulosa (6–8), and induction of ovulation (9). In the avian ovary, VIP induces accumulation of cAMP in theca (10) and granulosa (11, 12), inhibits plasminogen activator activity in granulosa (11), and stimulates production of androstenedione in theca (10) and progesterone in granulosa (11, 12). Immunoreactive VIP is localized in nerve fibers within theca but not in granulosa of the hen ovary (12). The present experiments were performed to demonstrate the presence of VIP receptor in the ovarian follicle of the hen, and to elucidate changes in the receptor binding during the ovulatory cycle.

Materials and Methods

Animals and Tissues. White Leghorn hens (18 months of age; 1.7 to 2.0 kg body wt) laying three to six sequential eggs with a 1-day pause between sequences for more than 2 weeks were used. They were obtained from a flock of approximately 1500 birds kept in individual cages under 14 hr (05:00 to 19:00 hr) light/day with feed and water provided for *ad libitum* consumption. The hens were killed by decapitation at 10:00 hr without regard to the time of ovulation (10 birds in each sample) to examine the binding reversibility, specificity, affinity, and capacity of cVIP binding component in ovarian follicles. To examine changes in the binding during an ovulatory cycle, the hens were sacrificed at five different times (16, 8, 6, 4, and 2 hr) before ovulation (08:00 hr) of the second ovum of the sequence (five birds/sample at each time). From the ovary of the hens, the largest (F_1), the second largest (F_2), and the third largest (F_3) follicles were excised and placed in ice-cold saline. Outer fibrous tissues were removed, and theca and granulosa layers were separated (13). They were washed in ice-cold saline to remove adhering yolk, blotted with a filter paper, weighed, and used immediately for preparation of membrane fraction.

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Preparation of Membrane Fraction. All steps were performed at 4°C. The tissues were homogenized in 3 v/w TE buffer (50 m*M* Tris-HCl, 2 m*M* EDTA, pH 7.4) containing 0.25 *M* sucrose by using a Potter-Elvehjem glass teflon homogenizer with 10 strokes while cooling in an ice-water bath. The homogenate was centrifuged at 700*g* for 10 min at 4°C, and the supernatant was obtained. The precipitate was resuspended in the buffer, rehomogenized, and centrifuged again. The pooled supernatants were centrifuged at 12,000*g* for 30 min at 4°C. The precipitate was washed twice with TE buffer not containing sucrose, and the precipitate was suspended in TE buffer and used as a membrane fraction. The fraction was stored at −80°C until assayed. The protein concentration of the fraction was measured by the method of Lowry *et al.* (14) using BSA (Fraction V; Sigma Chemical Co., St. Louis, MO) as a standard.

Radioiodination of cVIP. cVIP (Applied Biosystem, San Francisco, CA) was labeled with NA-^{125}I (Amersham International, Buckinghamshire, United Kingdom) using the iodogen method (15, 16) and chromatographed on a 1 × 100 cm column of Sephadex G-25 (superfine type; Pharmacia LKB, Uppsala, Sweden) eluted with 0.1 *M* acetic acid containing 0.1% BSA. Two clearly separated radioactive peaks prior to free ^{125}I peak were obtained. The first peak bound well to the membrane fraction, and the percentage of nonspecific binding to total binding was less than 20%. The second peak bound poorly. The first peak was used for binding assays within 40 days after radioiodination. The specific activity of ^{125}I-cVIP as monoiodo cVIP was calculated to be 610 to 840 Ci/mmol assuming a complete recovery of the peptide.

Binding Assay. The specific binding of ^{125}I-cVIP was measured by the use of the method of Gonzales *et al.* (15, 16) with modifications. The diluent used was TE buffer. Aliquots of the membrane fraction (5 μg protein/tube) were incubated at 4°C for 4 hr with ^{125}I-cVIP (0.06 to 1.4 n*M*) in the absence (total binding) or presence (nonspecific binding) of a 100-fold molar excess of unlabeled cVIP in a total volume of 300 μl. In the binding assay, 1.5-ml polypropylene microfuge tubes (Iwaki, Chiba, Japan) pretreated with TE buffer containing 2% BSA for 2 days at 4°C were used. For binding specificity, samples were incubated with ^{125}I-cVIP (0.4 n*M*) in the absence or presence of various molar excesses (0.4, 4, 40, or 400 n*M*) of unlabeled competitors. The unlabeled competitors used were cVIP, chicken secretin (Secretin; Peninsula Lab. Inc., Belmont, IL), rat peptide histidine isoleucine (PHI; Peninsula Lab. Inc., Belmont, IL), human glucagon (Glucagon; Peninsula Lab. Inc., Belmont, IL) and rat growth hormone releasing factor (GRF; Peninsula Lab. Inc., Belmont, IL). After the incubation, the tubes were centrifuged at 10,000*g* for 20 min at 4°C. The precipitated pellet was rinsed with 1 ml of ice-cold TE buffer, centrifuged again (10,000*g*, 20 min, 4°C), and its radioactivity was measured by a gamma counter (Cobra Model B5002; Packard Instrument Co., Downers Grove, IL). The counting efficiency for ^{125}I was 67% to 80%. Specific binding was obtained by subtracting nonspecific binding from total binding, and expressed as moles per milligram protein. The equilibrium dissociation constant (K_d) and the maximum binding capacity (B_{max}) were determined by the method of Scatchard (17).

Statistical Analyses. Kinetic data were analyzed by the method of Bylund and Yamamura (18), using pseudo-first-order conditions to estimate the association rate constant (K_{+1}) and addition of a large excess of unlabeled ligand to estimate the dissociation rate constant (K_{-1}).

The data, except for Fig. 3 and 4, were expressed as the mean ± SEM of three to five separate pools of samples. For comparisons among more than two groups, the data were analyzed by one-way analysis of variance (ANOVA) (19). When significant ($P < 0.05$) effects were found, Tukey's multiple range test was used to separate means (19). P values <0.05 were considered significant.

Results

Relationship of Specific ^{125}I-cVIP Binding to Incubation Temperature and Period. The specific binding at 4°C was found to increase during the first 4 hr of incubation and was then maintained up to 16 hr (Fig. 1). At 30°C, the binding increased during the first 30 min and then decreased. The maximum binding was almost equal at the two different temperatures.

Relationship of Specific ^{125}I-cVIP Binding to Protein Concentration of Membrane Fraction. The specific binding increased linearly with the increase in

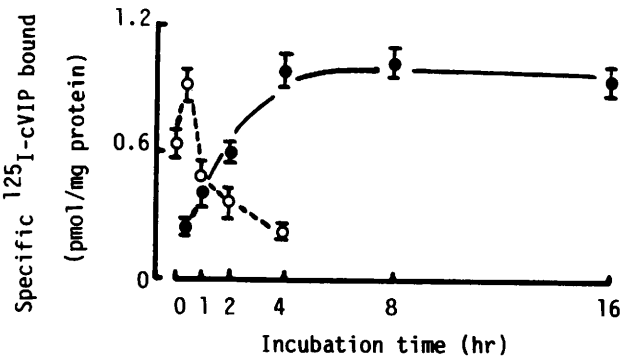

Figure 1. Time course of specific ^{125}I-chicken vasoactive intestinal peptide (cVIP) binding at 4°C (●) or 30°C (○). Membrane fractions (5 μg protein/tube) of granulosa cells of the largest preovulatory follicle of the hen were incubated with 1 n*M* ^{125}I-cVIP in the absence or presence of a 100-fold molar excess of unlabeled cVIP. Each point represents the mean ± SEM of three separate pools of samples.

the protein concentration from 1.25 to 20 μg per tube when incubated at 4°C for 4 hr.

Kinetic Analysis of Radioiodinated cVIP. A decrease in the specific ^{125}I-cVIP binding occurred upon addition of a large excess of unlabeled cVIP (Fig. 2). The association rate constant (K_{+1}) was 0.0090 ± 0.0003 nM^{-1} min^{-1}. Specific ^{125}I-cVIP binding was reduced ($t_{1/2}$ = 73 ± 6 min) by the addition of a large excess of unlabeled cVIP. The rate constant for dissociation (K_{-1}) determined from the pseudo-first-order equation was 0.0037 ± 0.0001 min^{-1}. The kinetic dissociation constant (K_d) for ^{125}I-cVIP calculated from the ratio K_{-1}/K_{+1} was 0.41 ± 0.03 nM (n = 5).

Binding Specificity. ^{125}I-cVIP binding in the membrane fraction of granulosa cells of F_1 was markedly reduced by the presence of a 100-fold molar excess of unlabeled cVIP, but little affected by the presence of an equivalent molar concentration of unlabeled GRF, PHI, Secretin, or Glucagon (Fig. 3). GRF, PHI, and Secretin reduced the binding a little (10% to 30%) when a 1000-fold molar excess was used.

Binding Affinity and Capacity. Specific ^{125}I-cVIP binding increased when increasing amounts of ^{125}I-cVIP were added (i.e., when the amount of free ^{125}I-cVIP was increased), and was saturable at about 0.7 nM (Fig. 4). A linear relationship between the amount of ^{125}I-cVIP specifically bound and the ratio of ^{125}I-cVIP specifically bound to free ^{125}I-cVIP (B/F) was observed (Fig. 4), indicating a single class of binding sites. The value of K_d, B_{max} and correlation coefficient (r) between B/F and specific ^{125}I-cVIP binding (calculated from the data in Fig. 4) was 0.36 nM (K_d), 1.47 pmol/mg protein (B_{max}), and −0.980 (r), respectively.

Table I lists the average K_d and B_{max} values determined by Scatchard analysis. The K_d value was greater in F_3 than in F_1 and F_2 ($P < 0.01$). The B_{max} value was greater in F_2 and F_3 than in F_1 ($P < 0.01$).

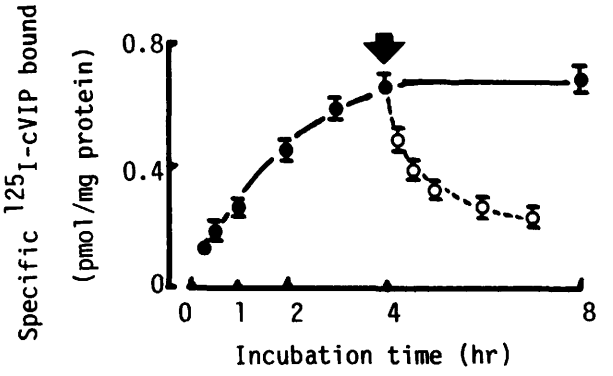

Figure 2. Association (●) and dissociation (○) of the specific ^{125}I-chicken vasoactive intestinal peptide (cVIP) binding. Membrane fractions (5 μg protein/tube) of granulosa cells of the largest preovulatory follicle of the hens were incubated at 4°C with 0.4 nM ^{125}I-cVIP in the absence or presence of a 100-fold molar excess of unlabeled cVIP. The arrow indicates the addition of 1 μM (1.1 μg per 20 μl assay buffer) of unlabeled cVIP. Each point represents the mean ± SEM of five separate pools of samples. Standard errors not shown fall within the point as drawn.

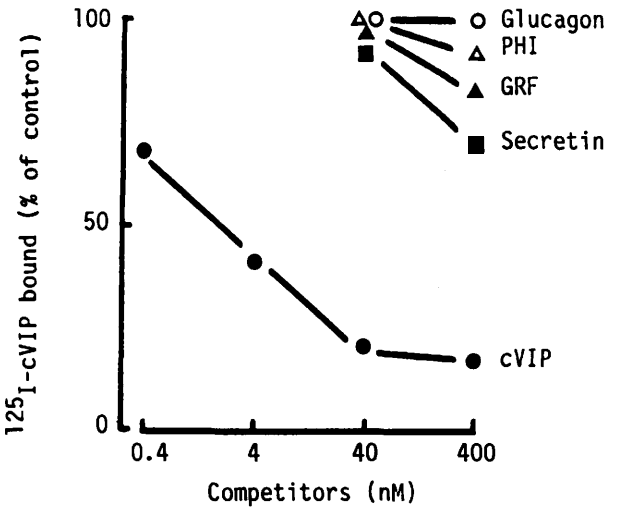

Figure 3. Competition for ^{125}I-chicken vasoactive intestinal peptide (cVIP) binding in membrane fractions of granulosa cells of the largest preovulatory follicle of the hen. Samples (5 μg protein/tube) were incubated at 4°C for 4 hr with 0.4 nM ^{125}I-cVIP in the absence (control) or presence of various molar excesses of unlabeled competitors. The ^{125}I-cVIP binding in the control was 752 fmol/mg protein. Each point represents the mean of two separate pools of samples. The competitors were: cVIP (●), chicken secretin (Secretin; ■), rat peptide histidine isoleucine (PHI: △), human glucagon (Glucagon; ○), rat growth hormone-releasing factor (GRF; ▲).

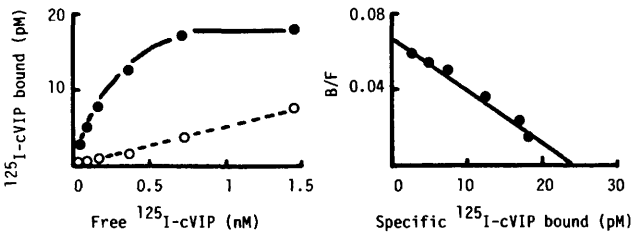

Figure 4. Saturation curves and Scatchard plots of specific ^{125}I-chicken vasoactive intestinal peptide (cVIP) binding in membrane fractions of granulosa cells of the largest preovulatory follicle of the hen. Samples (5 μg protein/tube) were incubated at 4°C for 4 hr with various concentrations (0.06 to 1.4 nM) of ^{125}I-cVIP in the absence or presence of a 100-fold molar excess of unlabeled cVIP. Each point represents the mean of duplicate determinations on one pooled sample. (●) specific binding, (○) nonspecific binding, B = specific ^{125}I-cVIP bound, F = free ^{125}I-cVIP.

Changes During Ovulatory Cycle. Figure 5 shows K_d and B_{max} values of F_1, F_2 and F_3 during the ovulatory cycle. In F_1, the K_d value increased 4 to 2 hr before ovulation ($P < 0.01$). The B_{max} value decreased 8 to 4 hr before, and increased 4 to 2 hr before ovulation ($P < 0.01$). In F_2 and F_3, no change in the K_d and B_{max} values was observed during the ovulatory cycle.

Discussion

The membrane fraction of granulosa cells of the hen was found to possess a specific VIP-binding com-

Table I. The Equilibrium Dissociation Constant (K_d) and the Maximum Binding Capacity (B_{max}) of the Chicken Vasoactive Intestinal Peptide Binding Component of Membrane Fraction of Granulosa Cells of the Largest (F_1), the Second Largest (F_2), and the Third Largest (F_3) Follicles of the Hen

Follicle	K_d (n*M*)	B_{max} (pmol/mg protein)
F_1	0.38 ± 0.04[a]	1.48 ± 0.18[a]
F_2	0.48 ± 0.04[a]	2.26 ± 0.11[b]
F_3	0.72 ± 0.05[b]	2.38 ± 0.12[b]

Note. Samples (5 μg protein/tube) were incubated at 4°C for 4 hr with ^{125}I-cVIP (0.06 to 1.4 n*M*) in the absence or presence of a 100-fold molar excess of unlabeled cVIP. The K_d and B_{max} were obtained by Scatchard analysis. The correlation coefficient (*r*) between specific binding per free ^{125}I-cVIP (B/F) and specific ^{125}I-cVIP binding was −0.973 to −0.991. Values are mean ± SEM of four separate pools of samples. Means in the same column with no common superscripts are significantly different ($P < 0.01$) by Tukey's multiple range test.

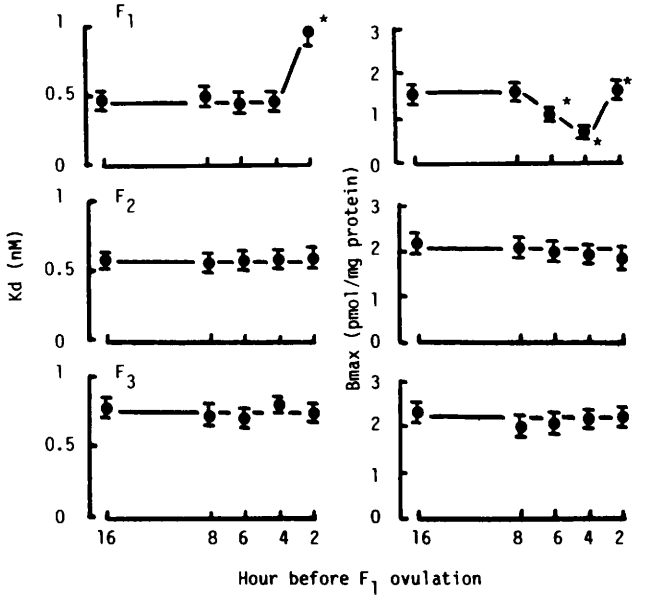

Figure 5. The equilibrium dissociation constant (K_d) and the maximum binding capacity (B_{max}) of vasoactive intestinal peptide (VIP) receptor binding in membrane fractions of granulosa cells of the largest (F_1), the second largest (F_2), and the third largest (F_3) follicles at various times before ovulation. Samples (5 μg protein/tube) were incubated at 4°C for 4 hr with ^{125}I-chicken VIP (^{125}I-cVIP; 0.06 to 1.4 n*M*) in the absence or presence of a 100-fold molar excess of unlabeled cVIP. The K_d and B_{max} were obtained by Scatchard analysis. The correlation coefficient (*r*) between bound per free (B/F) and specific binding was −0.971 to −0.990. Values are the mean ± SEM of four separate pools of samples. *Significantly different from the preceding value ($P < 0.01$) by Tukey's multiple range test.

ponent showing a reversible binding, binding specificity, high affinity, and limited capacity (Fig. 2, 3, and 4). These findings may indicate that the binding component is regarded as being a receptor for cVIP. In mammals, the primary structure of the N terminus 28 residues of pituitary adenylate cyclase-activating polypeptide 38 (PACAP 38) shows a 68% amino acid sequence homology with mammalian VIP (20). The PACAP specific receptor does not show competition with mammalian VIP (21), and is postulated to be obviously distinct from VIP receptor (22). We have investigated the membrane fraction of theca of F_1, F_2, and F_3, but no specific VIP binding was detectable following incubations in various protein concentrations (5, 10, 20, 40, and 80 μg/tube) at 4° and 30°C for up to 16 hr. VIP has been reported to be localized in theca and not in granulosa of the hen ovarian follicle (12). It is assumed that VIP liberated from the theca may act on the granulosa cells through binding to its receptor.

The value of K_d obtained from kinetic analysis was in close agreement with that obtained from Scatchard analysis. The binding site was of a single class. The value of K_d is almost the same as that reported on the chicken pineal (23), hypothalamus (16), and pituitary (15); the rat brain (24); and the cat renal outer medulla (25). However, a different value was reported on the chicken thymus and bursa of fabricius (26), the guinea pig brain (27); the rat brain (28), pineal (29), blood vessels (30), and uterus (31); and the feline kidney (25). In these tissues, the binding component possesses two distinct binding sites. A difference in the property of VIP receptor between animals and between tissues seems to exist. The B_{max} value obtained in the present study is of a similar order to that reported on the hen pituitary (15) and hypothalamus (16), and on the high-affinity binding component in various VIP-responsive tissues of mammals (25, 27–31).

A higher affinity (i.e., a smaller K_d value) of the VIP receptor was found in a larger follicle, and a greater binding capacity was found in a smaller follicle (Table I). This finding suggests that the property of VIP receptor in the hen ovarian follicle is related to the follicular growth. The affinity and capacity in the largest preovulatory follicle, F_1, were found to change during the ovulatory cycle shortly before ovulation but not in the smaller follicles, F_2 and F_3 (Fig. 5). The drastic changes before ovulation were shown at a time corresponding to the time of increase in the plasma level of progesterone (32, 33). It is suggested that the change in the VIP receptor binding in F_1 may be concerned in the induction of ovulation as suggested in rats (9). In the hen granulosa cells, VIP is known to stimulate the production of progesterone (11, 12), which is an ovulation-inducing factor (34). However, it seems unlikely that the difference in the binding affinity and capacity of VIP receptor between follicles of different size and the changes found only in F_1 before ovulation are related to the progesterone production in the granulosa cells, because a recent report by Johnson *et al.* (12) indicates that the stimulating action of VIP on the granulosa cells *in vitro* for the progesterone production is less in F_1 than in other smaller

follicles. The physiological significance of the VIP receptor binding in the hen granulosa cells needs further investigations.

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