Appearance of an arginine vasotocin receptor of large molecular size in the uterus (shell gland) of the hen at oviposition

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The molecular mass of the arginine vasotocin receptor in membrane fractions of the uterus (shell gland) of hens was estimated by autoradiography of [¹²⁵I]-labelled arginine vasotocin binding protein after SDS-PAGE. A band of approximately 67 kDa was found in all of the samples taken from laying hens before and after oviposition and from nonlaying hens. In addition to this band, another band of approximately 95 kDa was found in samples from laying hens taken immediately before and after oviposition. Both bands were reduced by the presence of unlabelled arginine vasotocin or mesotocin, but the reduction was greater by arginine vasotocin than by mesotocin. These two bands were not reduced by chicken luteinizing hormone-releasing hormone-I (Gln⁸-GnRH), chicken luteinizing hormone-releasing hormone-II (His⁵,Trp⁷,Tyr⁸-GnRH) and chicken angiotensin-II (Val⁵-angiotensin-II). The appearance of the arginine vasotocin receptor of the larger molecular size may be related to oviposition in hens.

Introduction

The avian neurohypophyseal hormone, arginine vasotocin (AVT) induces oviposition (expulsion of egg) (Rzasa and Ewy, 1970) by causing the contraction of the uterus (shell gland) in the hen (Munsick *et al.*, 1960). The presence of a receptor for AVT in the uterus of the hen was demonstrated by Takahashi *et al.* (1992), and the AVT receptor binding affinity increases and capacity decreases at the time of oviposition (Takahashi *et al.*, 1994). However, the cause of the increase in the binding affinity of the receptor has not been elucidated. Brennan (1988) reported that the binding affinity of the insulin receptor in the brain of rats changes together with a change in the molecular size of the receptor during fetal development in the uterus. The present study was performed to determine whether the AVT receptor of the hen uterus changes at the time of oviposition.

Materials and Methods

Animals and tissue preparations

White Leghorn hens (18–24 months of age; 1.8–2.2 kg body weight), kept under a lighting regimen of 14 h light (05:00–19:00 h) per day with feed and water provided *ad libitum*, were used. Uterine myometrial tissues were obtained from laying hens at four different times: 3 h before, within 5 min before, within 5 min after, and 1 h after oviposition of the first egg of a sequence occurring at about 07:00 h. At each time, four hens were served. The tissues were also

obtained from four nonlaying hens that had not laid an egg for at least 10 days. In the nonlaying hens, the ovarian mass was less than 8.8 g and the oviduct mass was less than 11.2 g. The serum concentrations of progesterone and oestradiol, as measured by radioimmunoassay (Shodono *et al.*, 1975), were 312 ± 13 pmol l⁻¹ ($n = 51$) and 294 ± 12 pmol l⁻¹ ($n = 51$), respectively. Membrane fractions of the tissues were prepared by the method reported by Takahashi *et al.* (1992). Protein concentrations of the fractions were determined by the method of Lowry *et al.* (1951) using BSA (fraction V; Seikagaku Kogyo Co., Ltd, Tokyo) as the standard.

[¹²⁵I]-labelled AVT binding and cross-linking

AVT (Bachem Inc., Torrance, CA) was labelled with ¹²⁵I by the use of Iodogen[®] (Sigma Chem. Co., St Louis, MO). The specific activity of the [¹²⁵I]-labelled AVT was 1877 Ci mmol⁻¹. Methods used for labelling and the determination of specific activity were described by Takahashi *et al.* (1992). The membrane fractions (30 µg per tube) were incubated at 30°C for 16 h with 1 nmol [¹²⁵I]-labelled AVT l⁻¹ in TE buffer (50 mmol Tris-HCl l⁻¹, 2 mmol EDTA l⁻¹, pH 7.4) in the presence or absence of 10 µmol unlabelled AVT l⁻¹, mesotocin (MT; Bachem), chicken (c) luteinizing hormone-releasing hormone-I (cLHRH-I; Gln⁸-GnRH; Peninsula Lab., Inc., Belmont, CA) cLHRH-II (His⁵,Trp⁷,Tyr⁸-GnRH; Bachem) and cAngiotensin-II (Val⁵-angiotensin-II; Bachem) in a total volume of 300 µl. Bound [¹²⁵I]-labelled AVT was separated from free [¹²⁵I]-labelled AVT by centrifugation (10 000 g, 10 min, 4°C) and washed by the same centrifugation. The precipitate was suspended in 200 µl TE buffer. Cross-linking of

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AVT receptor to the labelled AVT was carried out for 15 min at 4°C following the addition of 20 µl 0.11 mmol disuccinimidyl suberate l^{-1} (DSS; Pierce, Rockford, IL) dissolved freshly in dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd, Osaka) (Brennan, 1988; Bruno and Berelowitz, 1989; Rosenfeld *et al.*, 1989). The reaction was terminated by adding 280 µl stop buffer (100 mmol Tris-HCl l^{-1} , 10 mmol EDTA l^{-1} , pH 7.4) (Rosenfeld *et al.*, 1989), and the mixture was centrifuged (10 000 g, 10 min, 4°C). The precipitate was suspended in 25 µl of the sample buffer, 62.5 mmol Tris-HCl l^{-1} , 2% (w/v) sodium dodecyl sulfate (SDS; Kishida Chemical Co., Ltd, Osaka), 1% (v/v) Triton X-100 (Nacalai Tesque, Inc., Kyoto), 20% glycerol, 0.01% (w/v) bromophenol blue (BPB; Nacalai), pH 6.8, and shaken for 60 min at 4°C (Swedlund and Rosenzweig, 1990). After centrifugation (10 000 g, 10 min, 4°C), the supernatant was subjected to the SDS-polyacrylamide gel electrophoresis (PAGE). Relative migrations of specific bands were not altered by the presence of 2-mercaptoethanol (Nacalai) at the concentration of 2% (v/v) in the sample buffer.

SDS-PAGE and autoradiography

The SDS-PAGE was run with 15 mA for 1.5 h according to the method of Laemmli (1970) using 5–20% gradient polyacrylamide slab gels (1 mm thickness, 70 mm × 90 mm; SPG-520L PAGEL[®]; Atto Corp., Tokyo). After the electrophoresis, the gel was soaked in 10% acetic acid for 30 min. Both sides of the gels were covered with a polyester film (100 µm thickness), and the gels were placed under Imaging Plate (Fuji Photo Film Co., Ltd, Tokyo) (Sonoda *et al.*, 1983; Amemiya and Miyahara, 1988) for 16 h at room temperature. The sensitivity of the Imaging Plate for the radiation from ^{125}I is known to be approximately 4000 times higher than usual X-ray films, and the photostimulated luminescence increases linearly with an increase in the radioactivity from 1 to 10 000 d.p.m. mm^{-2} (Fuji Technical Information, 1993). Autoradiograms were obtained by the use of Fujix Bio-Imaging Analyzer (BAS2000; Fuji). The apparent molecular weight was determined by using the prestained protein molecular weight standards (Life Technologies, Inc., Gaithersburg, MD), lysozyme (MW: 15 550), β -lactoglobulin (MW: 18 200), carbonic anhydrase (MW: 28 050), ovalbumin (MW: 44 350), BSA (MW: 71 150), phosphorylase B (MW: 112 400) and myosin with H-chain (MW: 236 700).

AVT receptor binding assay

The binding assay used for the AVT receptor was reported by Takahashi *et al.* (1992). The equilibrium dissociation constant (K_d), an index of binding affinity, and the maximum binding capacity (B_{max}) were estimated by Scatchard analysis (Scatchard, 1949). The samples that were used for SDS-PAGE were also used in the binding assays. In each group of samples, four replicate determinations were performed.

Statistical analyses

Data were analysed by one-way ANOVA (Snedecor and Cochran, 1967). When a significant effect was found

Fig. 1. Autoradiography of SDS-PAGE of the membrane fraction of the uterine myometrium of laying and nonlaying hens. (a) Competition in laying hens 5 min before oviposition. AVT: arginine vasotocin; MT: mesotocin; cLHRH-I: chicken luteinizing hormone-releasing hormone-I, Gln^8 -GnRH; cLHRH-II: His^5, Trp^7, Tyr^8 -GnRH; cAngiotensin-II: Val^5 -angiotensin-II. (b) Laying hens at different times (-3: 3 h before oviposition, -0: within 5 min before oviposition, +0: within 5 min after oviposition, +1: 1 h after oviposition) and non-laying hens (NL). -: Without unlabelled AVT; +: with unlabelled AVT.

($P \leq 0.05$), Tukey's multiple range test (Tukey, 1953) was used to compare means of five groups in the present study.

Results

Three distinct dense bands were observed in the autoradiogram of SDS-PAGE after cross-linking of [^{125}I]-labelled AVT in the membrane fraction of the uterus of laying hens within 5 min before oviposition (Fig. 1a). The apparent molecular mass of

Table 1. Molecular size, equilibrium dissociation constant (K_d) and maximum binding capacity (B_{max}) of arginine vasotocin (AVT) receptor of the uterine myometrium in laying hens before and after oviposition, and in nonlaying hens

Hen	Time (h) before (–) and after (+) oviposition	Molecular mass		K_d (nmol l ⁻¹)	B_{max} (pmol mg ⁻¹ protein)
		67 kDa	95 kDa		
Laying	– 3	+	ND	0.83 ± 0.06 ^b	2.89 ± 0.11 ^d
	– 0 (within 5 min)	+	+	0.50 ± 0.02 ^a	0.92 ± 0.03 ^b
	+ 0 (within 5 min)	+	+	0.34 ± 0.03 ^a	0.39 ± 0.01 ^a
	+ 1	+	ND	0.88 ± 0.03 ^b	1.96 ± 0.04 ^c
Nonlaying	–	+	ND	0.90 ± 0.02 ^b	1.78 ± 0.02 ^c

The value of molecular size represents the mean of four birds. The same samples were assayed for [¹²⁵I]-labelled AVT binding. + : detectable; ND: not detectable. The K_d and B_{max} values obtained by Scatchard analysis are the means ± SEM of four birds. Values with different letters are significantly different ($P < 0.01$).

the three bands was estimated to be 21, 67 and 95 kDa, respectively. When a 10 000-fold molar excess of unlabelled AVT was present together with the [¹²⁵I]-labelled AVT in the cross-linking, the density of 67 and 95 kDa bands was reduced but not of 21 kDa band. The amount of [¹²⁵I]-labelled AVT binding in the 67 kDa band, calculated from the value of the photo-stimulated luminescence of the Bio-Imaging Analyzer, was 1.42 fmol in the absence of unlabelled AVT or MT, 0.27 fmol in the presence of unlabelled AVT, and 0.63 fmol in the presence of unlabelled MT, respectively. In the 95 kDa band, it was 0.61 fmol in the absence of unlabelled AVT or MT, 0.11 fmol in the presence of unlabelled AVT, and 0.25 fmol in the presence of unlabelled MT. In the presence of cLHRH-I, cLHRH-II and cAngiotensin-II, the autoradiogram appeared to be the same as that in the absence of any competitor.

The 95 kDa band was not observed in the membrane fraction of the laying hen at 3 h before oviposition and at 1 h after oviposition, and also not in the nonlaying hen (Fig. 1b).

The K_d and B_{max} values of unseparated receptors were smaller in the hens within 5 min before or after oviposition than in the hens 3 h before or 1 h after oviposition, and also than in the nonlaying hens (Table 1).

Discussion

Among the three bands observed in the autoradiogram, the 67 kDa and 95 kDa bands may be the AVT receptor and the other band of 21 kDa may be non-specific binding component to [¹²⁵I]-labelled AVT, because both 67 kDa and 95 kDa bands were reduced by the presence of unlabelled AVT but the 21 kDa band was not. Both bands were reduced also by the presence of unlabelled MT, but the reduction as estimated by the density measurement was greater in the presence of AVT than in the presence of MT. The reduction was similar to our earlier findings in binding experiments (Takahashi *et al.*, 1992). The existence of receptors having two molecular sizes is known in the uterus of rabbits on oxytocin receptors in which the molecular size is 50 and 65 kDa (Hinko *et al.*, 1992). The 95 kDa AVT receptor may appear only at the time of oviposition in the laying hen, because the receptor of this molecular size was not found at 3 h before oviposition and 1 h

after oviposition in the laying hen and at a time in the nonlaying hen (Fig. 1b). The appearance of the larger molecular sized AVT receptor may be the cause of the increase in the binding affinity (decrease in the K_d value) of unseparated AVT receptors at the time of oviposition found in the present study as in our earlier findings (Takahashi *et al.*, 1994). In the mammalian uterus, neither the change in the binding affinity of receptor for oxytocin nor the appearance of new receptor of different molecular size at parturition has been reported. In the hen uterus, minor contractions of uterine muscles are observable during the period when there is an egg in the uterus, and the contraction increases markedly just before oviposition (Shimada and Asai, 1978). It seems likely that the two AVT receptors may control the phased contraction of the uterus in the hen.

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