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Changes in Calcitonin Receptor Binding in the Shell Gland of Laying Hens (*Gallus domesticus*) During the Oviposition Cycle

Teruko Ieda[1)], Tetsuya Takahashi[2)], Noboru Saito[1)], Tadashi Yasuoka[3)], Mitsuo Kawashima[2)], Tokukazu Izumi[4)] and Kiyoshi Shimada[1)]

[1)] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan
[2)] Faculty of Agriculture Science, Gifu University, Gifu 501-1193, Japan
[3)] School of Medicine, Gifu University, Gifu 500-8705, Japan
[4)] Department of Animal Reproduction, Ishikawa Agricultural College, Nonoichi-machi, Ishikawa 921-8836, Japan

This study was conducted to examine calcitonin (CT) receptor binding in the membrane fraction of the endometrium of the shell gland of laying hens by the use of [^{125}I] CT binding assays. Specific [^{125}I] CT binding was found in the endometrium of the shell gland and increased during the first 1 h of incubation and then reached a plateau. Specific [^{125}I] CT binding increased in line with an increase in protein concentration from 20 to 70μg/tube. The membrane fraction had specific binding to [^{125}I] CT. The Scatchard plot analysis revealed a linear profile that indicated the presence of one type of receptor. In egg-laying hens, the equilibrium dissociation constant (Kd) and the maximum binding capacity (Bmax) of the endometrium in the shell gland started to increase soon after oviposition, reached a maximum 19 h before the next oviposition and remained high for 8-13 hours. Then it started to decrease 6 h before oviposition and reached a minimum at oviposition. In contrast, Kd and Bmax in nonlaying hens were constant throughout the 24 h period. These results suggest that CT may not act on the endometrium of the shell gland during most time of the shell calcification period but may act several hours during the last period of calcification as well as post-calcification period.

Key words : chicken, shell gland, calcitonin receptor, culcification

Introduction

Calcitonin (CT) is a polypeptide hormone consisting of 32 amino acids and a seven-membered ring at the N terminus. It is secreted from the ultimobrachial gland in birds or from the thyroid gland in mammals. In mammals, CT has been shown to regulate plasma Ca levels, because CT causes hypocalcemic action and inhibits osteoclastic bone resorption on the basis of its hypocalcemic action (Copp and Kline, 1989). In chickens, plasma CT levels positively correlated with dietary Ca intake and

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Correspondence and reprint requests should be addressed to Dr. Kiyoshi Shimada, Laboratory of Animal Physiology, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan. Fax : 052-789-4065. E-mail : kshimada@agr.nagoya-u.ac.jp

thus to circulating Ca levels (Taylor and Dacke, 1984) but the role of CT in the regulation of calcium in plasma are controversial. CT has been thought to act upon bone to decrease bone resorption and thereby decrease blood calcium and phosphate values. Belanger and Copp (1972) demonstrated that chronic administration of CT to cockerels caused decreased activity of osteocytes and osteoclasts, smaller osteocytic lacunae and increased bone density. CT also causes disappearance of ruffled borders in cultured medullary bone osteoclasts (Sugiyama and Kusuhara, 1996). These findings are consistent with the idea that CT downregulates plasma calcium under normal physiological conditions in the chick. In contrast, ultimobranchialectomy has no effect on bone or plasma calcium concentrations in young chicks (Brown *et al.* 1970) and dosing heavily fasted chicks with salmon CT *in vivo* caused inhibition of net ^{45}Ca uptatke in the femur (Ancill *et al.*, 1991). It is still obscure whether CT has any major role in bone calcium regulation in birds. Recently, we observed that the binding affinity of parathyoid hormone (PTH) receptors in the shell gland of the laying chickens increases during the period between 19 h before oviposition and immediately before oviposition with a concomitant decrease in the binding capacity (Ieda *et al.*, 2000). These changes correspond to the decrease in the ionized plasma calcium level possibly to due to increased calcium demand for the eggshell calcification (Yasuoka *et al.*, 1996). Since CT can be classically considered to be a physiological antagonist of PTH, it seems interesting to study the binding characteristics of CT receptors of shell gland of the chicken as a target tissue of CT. CT recptors have been found in the kidney and the bone of rats (Marx *et al.*, 1972) and in the kidney of chickens (Yasuoka *et al.*, 1998). Chickes have a very high levels of plasma CT compared to mammals (Kenny, 1971). In quail, CT levels in plasma varied during an ovulation cycle ; the levels significantly decrease at 5–22 after ovulation, corresponding to the time of shell calcification, indicating that CT may play a role on the calcium metabolism of the laying birds (Dacke *et al.*, 1972). Accordingly, this study aims to elucidate the binding property of CT receptors in the shell gland of laying hen and a possible involvement of CT on shell gland in association with egg shell formation.

Materials and Methods

Animals and tissues

White Leghorn hens (18–20 months of age ; 1.7–2.2 kg of body weight) were killed by decapitation at 10 : 00 h to examine the binding specificity, reversibility, affinity, and capacity of CT receptor (5 birds in each sample). The hens were also killed at seven different times (-22, -19, -11, -6, -3, -0 ; just before oviposition, +0 ; just after oviposition) during oviposition cycle (one bird per sample, four bird at each time) of the first egg of an egg-laying sequence to elucidate the change in the binding affinity and capacity of CT receptor in relation to egg shell formation. For comparison, nonlaying (molting) hens that had not laid an egg for at least 4 weeks prior to the experiments were killed at corresponding hours of the day (one bird per sample, four birds at each time). Food and water were given ad libitum but they have not laid eggs during the experimental periods. Endometrium of the shell gland was excised for the preparation

of membrane fractions.

Hormone preparations and chemicals

Chicken CT, chicken [Tyr^{36}] parathyroid hormone related peptide (1–36) amide (PTHrP), and chicken vasoactive intestinal peptide (VIP) were purchased from Peninsula Laboratories, Inc (San Carlos, CA), chicken angiotensin-II (Ang-II) was purchased from Bachem Inc. (Torrance, CA), and human insulin (Insulin) was purchased from Sigma Chemical Co. (St. Louis, MO). Na[^{125}I] was obtained from Amersham International plc (Buckinghamshire, UK). Iodogen (1, 3, 4, 6-tetrachloro-3a, 6a-diphenyl glycoluril) was purchased from Sigma Chemical Co. Sephadex G-25 (fine type) was obtained from Pharmacia LKB (Uppsala, Sweden), and bovine serum albumin (BSA) fraction V, pH 5.6, from Sigma Chemical Co., and BSA fraction V pH 7.0, from Seikagaku Corp. (Tokyo, Japan). All chemicals used were of analytical grade, obtained from Kishida Chemical Co., Ltd. (Osaka, Japan) or Nacalai Tesque, Inc. (Kyoto, Japan), unless otherwise stated.

Preparation of membrane fractions

Membrane fractions were prepared by using the method of Yasuoka *et al.* (1996) with slight modifications. All procedures were performed in a cold room at 4℃. The tissues were rinsed with ice-cold saline, blotted with filter paper, weighed, and then minced. The endometrium was homogenized by using Ultra-Turrax homogenizer (Type 18–10 ; Ika Labortechnik, Janke & Kunkel GmbH & Co KG, Staufen, Germany) in 10 v/w of TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.4). The homogenization was carried out by two repeated bursts, each for 30 s, in an ice bath. Homogenates of each sample were centrifuged at 800 g for 10 min at 4℃, and the supernatant was obtained. The pellet was resuspended in the buffer, and centrifuged at 800 g for 10 min at 4℃. The supernatants were combined and centrifuged at 30,000 g for 30 min at 4℃. The pellet was suspended with 15 vol of TE buffer and then resuspended. The suspension was centrifuged at 30,000 g for 30 min at 4℃. The pellet was mixed with 3 vol of TE buffer and divided into 1 m*l* aliquot, stored at −70℃, and used as the membrane fraction. The protein concentration was measurel by the method of Lowry *et al.* (1951) using BSA (fraction V, pH 7.0) as a standard.

Radioiodination of CT

Chicken CT was iodinated with Na 125I using Iodogen (Salacinski *et al.*, 1981). The iodinated CT was separated from free Na[^{125}I] in the same way as described by Takahashi *et al.* (1992). The mixture was placed onto a 1×100 cm Sephadex G-25 column pretreated with 1% BSA (fraction V, pH 5.6) and eluted with 0.1 M acetic acid solution containing 0.1% BSA. The elution were fractionated in each 1 m*l* volume, and the radioactivity was counted by a Packard Cobra gamma counter (Packard Instrument Co., Meriden, CT). A clearly separated peak of radioactivity prior to the peak of free Na[^{125}I] was obtained. The peak bound well to the membrane fraction, and the percentage of nonspecific binding to total binding was $<20\%$. The radioactive peak of the elution was employed for binding assays. The specific activity of the radioligand was calculated to be 302–487 Ci/mmol. All experiments were performed within 40 days after radioiodination.

Binding assay

The membrane fractions (30μg protein/tube) were incubated with various concentrations (0.06–2 nM) of [^{125}I] CT in the presence or absence of 1μM of unlabeled CT in a total volume of 300μl. Incubations were performed at 4℃ for 2 h in duplicate. Polypropylene Eppendorf-type microfuge tubes (1.5 m*l* ; Iwaki, Chiba, Japan) pretreated with TE buffer supplemented with 1% BSA (fraction V, pH 7.0) were employed for the incubation. Immediately after the incubation, the tubes were centrifuged at 10,000 g for 10 min at 4℃. The precipitated pellet was rinsed wit 0.5 m*l* of TE buffer, followed by recentrifugation. The radioactivity was measured using a Packard Cobra gamma counter ; the counting efficiency for ^{125}I was 70–87%. Specific binding was obtained by subtracting the nonspecific binding (in the presence of unlabeled hormone) from the total binding (in the absence of unlabeled hormone) and expressed as femtomoles per milligram of protein. The equilibrium dissociation constant (Kd) and the maximum binding capacity (Bmax) were determined by the method of Scatchard (1949). Means of duplicate determinations were used as the points of the Scatchard analysis. Kinetic data were analyzed by the method of Bylund and Yamamura (1990). The pseudo-first-order condition was adopted to estimated the association rate constant (k_{+1}), and the data obtained by the addition of a large excess of unlabeled ligand were used to estimate the dissociation rate constant (k_{-1}).

Statistical analyses

Statistical comparison among the groups during the oviposition cycle was performed by one-way ANOVA (Snedecor and Cochran, 1980a). When ANOVA was significant ($P \leq 0.05$), the Tukey's multiple range test was applied for the post-hoc comparisons (Snedecor and Cochran, 1980b).

Results

Effect of incubation temperature and period, and membrane protein concentration on CT binding

The binding to [^{125}I] CT in membrane fractions of the shell gland at 4℃ increased during the first 1 h of incubation, and then reached a plateau up to 4 h (Fig. 1). A linear increase in the specific binding with the increase in the protein concentration from 20 to 70μg per tube was observed when incubated at 4℃ for 2 h (Fig. 2).

Binding specificity

The binding to [^{125}I] CT in membrane fractions of the shell gland was markedly reduced by the presence of a 100- to 1000-fold molar excess of unlabeled CT. A equivalent molar concentration of unlabeled CT reduced the binding about 50%. The binding was not reduced by the presence of a 10- to 1000-fold molar excess of unlabeled PTHrP, Ang-II, Ins, and VIP (Fig. 3).

Kinetic analysis

The specific [^{125}I] CT binding in the membrane fraction of shell gland of hens reached a steady-state at 60 min and was stable for up to 120 min (Fig. 4). The addition of large excess of unlabeled CT at 60 min caused a gradual decrease in specific [^{125}I] CT binding (Fig. 4). The association rate constant (k_{+1}) was 0.044$\pm$0.003

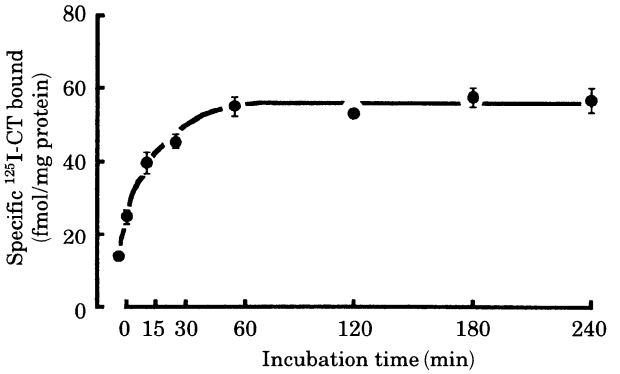

Fig. 1. Time course of the specific [^{125}I] CT binding in the membrane fraction of the endometrium in the shell gland of laying hens at 4℃ of incubation. Samples (30μg protein per tube) were incubated with 1 nM [^{125}I] CT in the absence or presence of a 1000-fold molar excess (1μM) of unlabeled CT, and the specific [^{125}I] CT binding was measured. Each point represents the mean±SEM of three separate pools of samples. Standard errors not shown fall within the point as drawn.

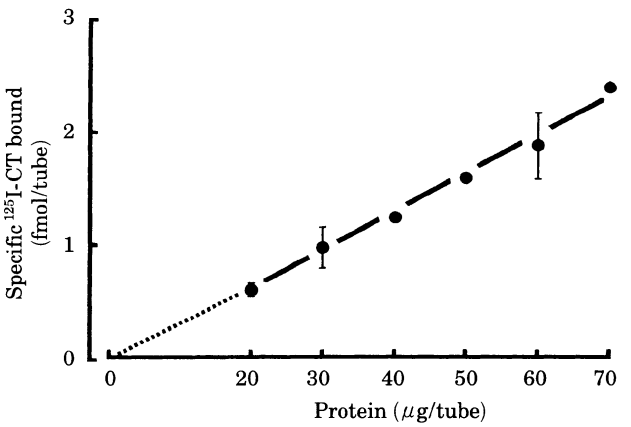

Fig. 2. Relationship of specific [^{125}I] CT binding to the protein concentration in the membrane fraction of the endometrium of the shell gland in laying hens. Samples (20 to 70μg protein per tube) were incubated at 4℃ for2 h with 1 nM [^{125}I] CT in the absence or presence of a 1000-fold molar excess (1μM) of unlabeled CT, and the specific [^{125}I] CT binding was measured. Each point represents the mean±SEM of three separate pools of samples. Standard errors not shown fall within the point as drawn.

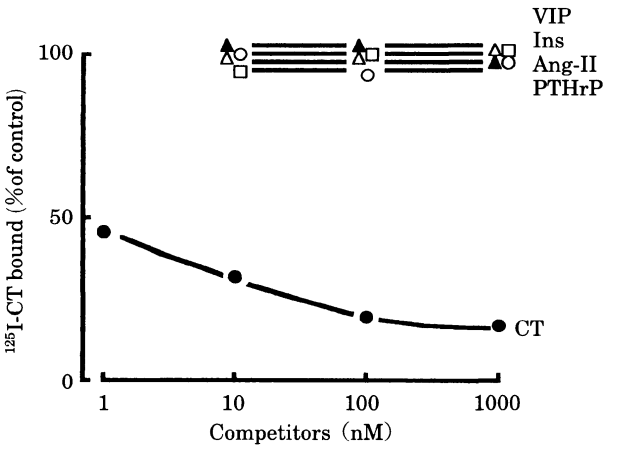

Fig. 3. Competition for [^{125}I] CT binding in the membrane fraction of the endometrium of the shell gland in laying hens. Samples (30μg protein per tube) were incubate at 4℃ for 2 h with 1 nM [^{125}I] CT in the absence (control) or presence of various fold molar excess of unlabeled chicken calcitonin (CT, closed circle), parathyroid hormone-related peptide (PTHrP, open circle), chicken angiotensisn-II (Ang-II, open triangle), chicken vasoacitive intestinal peptide (VIP, closed triangle), or insulin (Ins, open square). The amount of the [^{125}I] CT binding in control value was 67.4 fmol/mg protein. Each point represents the mean of two separate pools of samples.

nM^{-1} min^{-1} (n=5). Specific [^{125}I] CT binding was reduced ($t_{1/2}$=4.83±0.50 min, n =5) by the addition of a large excess of unlabeled CT. The rate constant for dissociation (k_{-1}) determined from the pseudo-first-order equation was 0.031±0.001 min^{-1} (n=5). The kinetic dissociation constant (Kd) for [^{125}I] CT calculated from the ratio k_{-1}/k_{+1} was 0.72±0.03 nM (n=5).

Binding affinity and capacity

The specific [^{125}I] CT binding in the membrane fraction of the shell gland increased when increasing amounts of [^{125}I] CT were added (i.e., when increasing the amount of free [^{125}I] CT), and was saturable at about 1.2 nM (Fig. 5). Scatchard analysis revealed a linear relationship between the amount of specific [^{125}I] CT binding and the ratio (bound/free) of specific [^{125}I] CT binding to free [^{125}I] CT (Fig. 5), indicating a single class of binding sites. The values of Kd, Bmax, and the correlation coefficient (g) between bound/free and specific [^{125}I] CT binding obtained from endometrium of the shell gland of five birds as used for the measurement of Scatchard analysis were 0.80 ±0.06 nM (Kd, mean±SEM), and 50.7±7.7 fmol/mg protein (Bmax), and −0.970 to −0.992 (γ), respectively (Table 1).

Changes in binding affinity and capacity

A decrease in the Kd value of the CT receptor in the shell gland was observed in

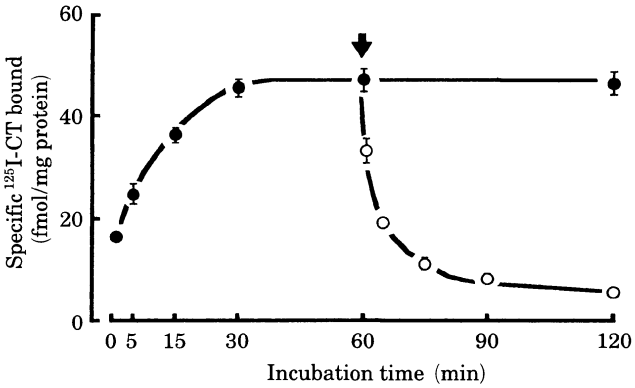

Fig. 4. Time course of the association (closed circle) and dissociation (open circle) of [^{125}I] CT in the membrane fraction of the endometrium of the shell gland in laying hens. Samples (30μg protein per tube) were incubated at 4℃ for various times with 1 nM [^{125}I] CT in the absence or presence of 1μM unlabeled CT, and the specific [^{125}I] CT binding was measured. The arrow indicates the addition of 7μM (1.2μg/50μl assay buffer) of unlabeled CT. Each point represents the mean±SEM of five separate pools of samples. Standard errors not shown fall within the point as drawn.

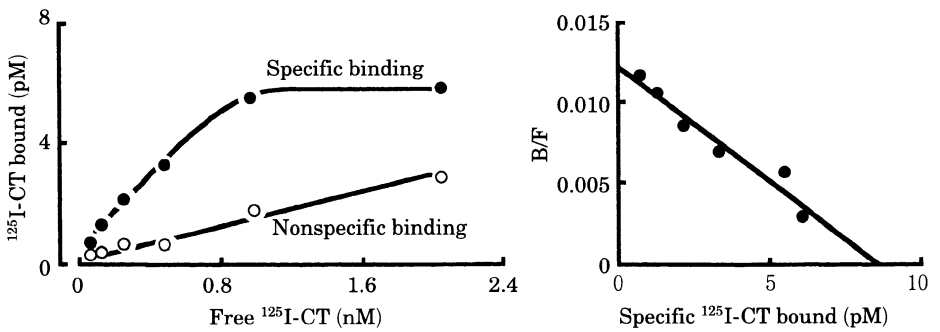

Fig. 5. Saturation curve and Scatchard plot of specific [^{125}I] CT binding in the membrane fraction of the endometrium of the shell gland in laying hens. Samples (30μg protein per tube) were incubated at 4℃ for 2 h with various concentrations (0.06-2 nM) of [^{125}I] CT in the absence or presence of 1μM unlabeled CT, and the specific [^{125}I] CT binding was measured. The value of Kd and Bmax calculated by the use of Scatchard analysis, and correlation coefficient (γ) between B/F and specific binding was 0.71 nM (Kd), 64.9 fmol/mg protein (Bmax), and −0.970 (γ), respectively. Each point represents the mean of two experiments in duplicate determinations from a pooled sample : Specific binding (close circle), nonspecific binding (open circle), (B) specific [^{125}I] CT bound, (F) free [^{125}I] CT.

endometrium of the shell gland of egg-laying hens at 6 h, 3 h, -0 h (within 5 min before oviposition) before oviposition, and 0 h (within 5 min after oviposition) ($P \leq 0.05$). No appreciable change was observed in nonlaying hens ($P > 0.05$, Fig. 6). A similar change in the Bmax value was also observed in the egg-laying hens ($P \leq 0.05$) but not in the nonlaying hen ($P > 0.05$, Fig. 6).

Discussion

The present study demonstrated the binding characteristics of the CT receptor of the shell gland in the chicken. Namely, the membrane fraction of endometrium of the shell gland of the hen was found to contain a binding component having a binding specificity (Fig. 3), a binding reversibility (Fig. 4) and a saturation binding (Fig. 5) as well as high affinity and limited capacity. The Kd (moles per liter) is an index of the binding affinity. The smaller Kd value shows the greater binding affinity with the order of 10^{-10} M. Values of Kd obtained from the kinetic analysis were in close agreement with those obtained by Scatchard analysis. The Kd value of the CT receptor of the shell gland in the laying hen showed a change during oviposition cycle but did not show any significant change in the nonlaying hen (Fig. 6). In the laying hen, the Kd value started to decrease 6 h before oviposition. The ionized calcium concentrations in plasma is low from 13 to 6 hours before oviposition in the laying hen (Etches, 1996 ; Yasuoka *et al.*, 1996) corresponding to the period of intense shell formation. During this period a large amount of calcium is transferred for the formation of the shell. But calcium secretion in the shell gland declines to basal secretion about 2 h before oviposition (Eastin and Spaziani, 1978). The decrease in a calcium deposition during shell formation is coincidental with an increase in the affinity (decrease in the Kd value) of CT receptors of the endometrium in the laying hen. These results may suggest that CT has a direct action on the shell gland near a cessation of shell formation and post-calcification period. A decrease in the Bmax value of the CT receptor in the endometrium was also noted during shell formation. The smaller Bmax value may be a consequence of the increase in CT binding to the receptor *in vivo* by an increase in the binding affinity of CT to the receptor. Recently, we also reported the changes in the Kd value of PTH receptors in the shell gland of the hen in relation to shell calcification. However, it should be noted that in our previous study the Kd value of PTH receptor showed a decrease during egg shell calcification (Ieda *et al.*, 2000). Such a mirror-image pattern of the Kd values of CT and PTH receptors during the shell calcification cycle indicates a physiological antagonist between CT and PTH acting on the shell gland. Although a precise mechanism of action of each hormone remains unclear, further studies of a competiting manner of hormones by targetting the same tissue, the endometrium of the shell gland of the laying hens, are warranted. In conclusion the present study demonstrated the presence of CT receptors in the shell gland of laying hens and the increase in CT binding affinity may be associated to the cessation of the calcium secretion in relation to calcification.

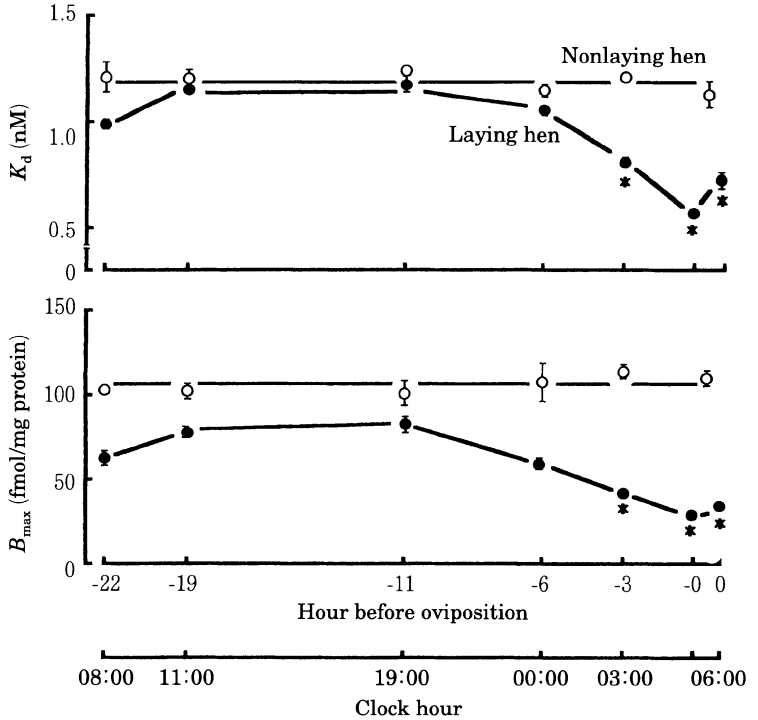

Fig. 6. The equilibrium dissociation constant (Kd) and the maximum binding capacity (Bmax) of the specific binding component of [^{125}I] CT in the membrane fraction of the endometrium of the shell gland of laying hens at various times before oviposition (close circle) and of nonlaying hens at corresponding times (open circle).

Samples (30μg protein per tube) were incubated at 4℃ for 2 h with [^{125}I] CT (0.06 to 2 nM) in the presence and absence of 1μM unlabeled CT, and the specific [^{125}I] CT binding was measured. The values of Kd and Bmax were obtained by Scatchard analysis.

Each point represents the mean±SEM of four separate samples. Asterisk indicate significantly different ($P \leq 0.05$) from values of points without asterisk within each group by Tukey's test. −0 h ; within 5 min before oviposition, 0 h ; within 5 min after oviposition.

References

Ancill AK, Bascal ZA, Whitaker G and Dacke CG. Calcitonin gene-related peptide promotes transient radiocalcium uptake into chick bone *in vivo*. Experimental Physiology, 76 : 143-146. 1991.

Belanger LF and Copp DH. The skeletal effects of prolonged calcitoninn administration in birds under various conditions. In : Calcium, parathyroid hormone and the calcitonins (R. Talmage, RV and Munson PL eds.). pp.41-50. Excerpta Medica. Amsterdam. 1972.

Brown DM, Perey DYE and Jowsey J. Effects of ultimobranchialectomy on bone composition and mineral metabolism in the chicken. Endocrinology, 87 : 1282-1291. 1970.

Bylund DB and Yamamura HI. Methods for receptor binding. In : Methods in Neurotransmitter Receptor Analysis (Yamamura HI and Enna SJ eds.). pp. 1-35. Raven Press. New York.

1990.

Copp DH and Kline LW. Calcitonin. In : Vertebrate Endocrinology Fundamentals and Biomedical Implications (Pang PKT and Schreibman MP eds.). Vol. 3. pp. 79-103. Academic Press. New York. 1989.

Dacke CG, Boelkins JN, Smith WK and Kenny AD. Plasma calcitonin levels in birds during the ovulation cycle. Journal of Endocrinology, 54 : 369-379. 1972.

Eastin WC Jr. and Spaziani E. On the control of calcium secretion in the avian shell gland (uterus). Biology of Reproduction, 19 : 493-504. 1978.

Etches RJ. Reproduction in Poultry. CAB International. Oxon. 1996.

Ieda T, Takahashi T, Saito N. Yasuoka T, Kawashima M. and Shimada K. Changes in parathyroid hormone-related peptide receptor binding in the shell gland of laying hens (*Gallus domesticus*) during the oviposition cycle. General and Comparative Endocrinology, 117 : 182-188. 2000.

Kenny AD. Determination of calcitonin in plasma by bioassay. Endocrinology, 89 : 1005-1013. 1971.

Lowry OH, Rosenbrough NJ, Farr AL and Rendall RJ. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry, 193 : 265-275. 1951.

Marx SJ, Woodard CJ and Aurbach GD. Calcitonin receptors of kidney and bone. Science, 178 : 999-1001. 1972.

Salacinski PRP, Mclean C, Sykes JEC, Clement-Jones VV and Lowry PJ. Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1, 3, 4, 6-tetrachloro-3α, 6 α-diphenyl glycoluril (Iodogen). Analytical Biochemistry, 117 : 136-146. 1981.

Scatchard G. The attractions of proteins for small molecules and ions. Annals New York Academy of Sciences, 51 : 660-671. 1949.

Snedecor GW and Cochran WG. One-way classifications : Analysis of variance. In : Statistical Methods (Snedecor GW and Cochran WG eds.). 7th ed. pp. 215-237. The Iowa State University Press. Ames. 1980a.

Snedecor GW and Cochran WG. Faileres in the assumptions. In : Statistical Methods (Snedecor GW and Cochran WG eds.). 7th ed. pp. 274-297. The Iowa State University Press, Ames. 1980b.

Sugiyama T and Kusuhara S. Morphoslogucal changes of osteoclasts on hen medullary bone during the egg-laying cycle and their regulation. In : Comparative Endocrinology of Calcium Regulating Hormones (Dacke CG, Dankes J, Flik G and Caple I eds.). Journal of Endocrinology Ltd., Bristol. 1996.

Takahashi T, Kawashima M, Kamiyoshi M and Tanaka K. Arginine vasotocin binding component in the uterus (shell gland) of the chicken. Acta Endocrinologica (Copenhagen), 127 : 179-184. 1992.

Taylor TG and Dacke CG. Calcium metabolism and its regulation. In : Physiology and biochemistry of the Domestic fowl. (Freeman BM ed.). Vol. 5. pp. 125-170. Academic Press. London. 1984.

Yasuoka T, Kawashima M, Takahashi T, Iwata A, Oka N and Tanaka K. Changes in parathyroid hormone receptor binding affinity during egg laying : Implications for calcium homeostasis in chicken. Journal of Bone and Mineral Research, 11 : 1913-1920. 1996.

Yasuoka T, Kawashima M, Takahashi T, Tatematsu N and Tanaka K Calcitonin receptor binding properties in bone and kidney of the chicken during the oviposition cycle. Journal of Bone and Mineral Research, 13 : 1412-1419. 1998.