Direct Action of Melatonin on Hen Ovarian Granulosa Cells to Lower Responsiveness to Luteinizing Hormone (44148)

TOHRU MURAYAMA,* MITSUO KAWASHIMA,†,[1] TETSUYA TAKAHASHI,† TADASHI YASUOKA,‡ TAKEHITO KUWAYAMA,* AND KATUHIDE TANAKA*

Department of Zootechnical Science, Tokyo University of Agriculture, Tokyo 156, Japan; Department of Biological Diversity and Resources,† Gifu University, Gifu 501-11, Japan; and Department of Oral and Maxillofacial Surgery,‡ Gifu University School of Medicine, Gifu 500, Japan*

Abstract. **The presence of a melatonin-binding component in the membrane fraction of the ovarian granulosa cells of hens that has characteristics of a receptor such as high affinity, limited capacity, reversible binding, and binding specificity was demonstrated by radioligand binding assays. When the cells were incubated *in vitro* with various concentrations of luteinizing hormone (LH) in the presence and absence of melatonin (1 and 5 n*M*), the dose-response curve for the LH-stimulated progesterone production was shifted to a higher concentration of LH: ED_{50} of LH was approximately two times greater in the presence of melatonin. The results suggest a direct action of melatonin on the ovarian granulosa cells of the hen to lower the responsiveness to LH for progesterone production.** [P.S.E.B.M. 1997, Vol 215]

Melatonin (*N*-acetyl-5-methoxytryptamine; MW = 232), the hormone of the pineal gland, is involved in the regulation of many physiological functions of various species of animals, including birds (1–3). It is generally accepted that melatonin influences reproduction *via* its action on the hypothalamus (4, 5) and hypophysis (6), and may modulate the secretion of the hypophyseal luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (7). Besides the central sites of action, the presence of a peripheral site of action is suggested by the fact that *in vitro* synthesis of steroids in the mammalian testis (8–10), ovarian granulosa (11, 12), and luteal cells (13, 14) are stimulated by the presence of melatonin, and that specific melatonin-binding sites are present in ovaries of chickens (15) and ducks (16), ovarian granulosa cells of humans (17), and testes of chickens (15), quail (18, 19), ducks (16), and rats (20).

In the avian ovary, progesterone is produced only in the granulosa cells (21, 22), and the production is stimulated by LH both *in vivo* (23) and *in vitro* (24). Responsiveness of the granulosa cells of the chicken to LH is known to differ between different follicles in size: greater in larger follicles (25, 26). Norepinephrine (27) has been reported to stimulate the production of progesterone in response to LH. No inhibitory factor of physiological significance has been demonstrated. The present study was performed to obtain evidence for a direct action of melatonin on the ovarian granulosa cells of the hen as indicated by the presence of a receptor and the effect of melatonin *in vitro* for LH-stimulated progesterone production.

Materials and Methods

Animals and Tissues. White Leghorn hens (18–20 months of age; 1.6–2.0 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 (0500 to 1900 hr) light/day with feed and water provided for *ad libitum* consumption. The hens were sacrificed by decapitation at 1200 hr, without regard to the time of ovulation, to examine the binding affinity and capacity, reversibility, and specificity of melatonin-binding component in ovarian follicles (40 birds in each sample), and to examine the effects of melatonin on progesterone production by LH in isolated granulosa cells (30 birds in each

[1] To whom requests for reprints should be addressed at Department of Biological Diversity and Resources, Gifu University, 1-1 Yanagido, Gifu 501-11, Japan.

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sample). 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 (28), washed in ice-cold saline to remove adhering yolk, and used immediately.

Preparation of Membrane Fraction and Binding Assay. All steps were performed at 4°C. The tissues were homogenized in 3 vol/wt TEMB buffer (25 m*M* Tris-HCl, 2 m*M* EDTA, 2 m*M* $MgCl_2$, 0.5 m*M* bacitracin, pH 7.4) containing 0.25 *M* sucrose 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 supernatants were combined and centrifuged at 12,000*g* for 30 min at 4°C. The precipitate was washed twice with TEMB buffer not containing sucrose, and the precipitate was suspended in TEMB buffer and used as the 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.* (29) using BSA (Fraction V; Sigma Chemical Co., St. Louis, MO) as a standard.

The specific binding of [^{3}H]melatonin was measured using a method reported earlier (30) with modifications. The diluent used was TEMB buffer. Aliquots of the membrane fraction (350 μg protein/tube) were incubated at 4°C for 60 min with 0.08–2.6 n*M* [^{3}H]melatonin (acetyl-5-methoxytryptamine, *N*-[methoxy-^{3}H]-: 83 Ci/mmol; E.I. Dupont de Nemours and Co. Inc., NEN Research Products, Boston, MA) in the absence (total binding) or presence (nonspecific binding) of a 1000-fold molar excess of unlabeled melatonin (Sigma) in a total volume of 300 μl. In the binding assay, 1.5-ml polypropylene microfuge tubes (Iwaki, Chiba, Japan) pretreated with TEMB buffer containing 2% BSA for 2 days at 4°C were used. To examine the binding specificity, samples were incubated with [^{3}H]melatonin (0.7 n*M*) in the absence or presence of various molar excesses (0.7, 7, 70, 700, and 7,000 n*M*) of unlabeled competitors. The unlabeled competitors used were melatonin, 6-hydroxymelatonin, 5-hydroxytryptophan, serotonin, *N*-acetylserotonin, 5-methoxytryptophol, and 5-methoxyindole acetic acid (all but melatonin from Sigma). After 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 TEMB buffer, centrifuged again (10,000*g*, 20 min, 4°C), and ACS-II scintillation fluid (Amersham International Plc, Buckinghamshire, England) added. Radioactivity was measured using a liquid scintillation analyzer (Packard 2500 TR; Packard Instrument Co., Downers Grove, IL) in which the counting efficiency for ^{3}H was 47%–53%. Specific binding was obtained by subtracting nonspecific binding from total binding, and expressed as moles per mg protein. The equilibrium dissociation constant (K_d) and the maximum binding capacity (B_{max}) were determined by the method of Scatchard (31).

Granulosa Cell Suspension and Incubation. Granulosa cells were isolated and incubated *in vitro* using the same method used in the incubation of pituitary cells (32, 33) with a slight modification. Collagenase treatment was carried out for 5 min at 37°C with HEPES (25 m*M* HEPES, 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 10 m*M* glucose, pH 7.4) buffer containing 0.08% collagenase and 0.25% BSA. Cells were collected by centrifugation (100*g*, 5 min) and washed twice with Romanoff's avian Ringer salt solution (RRA) (34). The washed cells were suspended in the incubation medium (RRA containing 0.25% BSA). Concentrations of the cells in the medium were determined by counting with a hemacytometer and adjusted to 2×10^6 viable cells/ml by dilution with the incubation medium. Viability of the cells was estimated to be over 95% by trypan blue staining (35). The granulosa cell suspensions (0.5 ml: 1×10^6 cells) were placed in polystyrene tubes (16 × 125 mm; Falcon Plastics, Los Angeles, CA), each in duplicate, to which 0.5 ml incubation medium was added. The tubes were preincubated at 40.5°C for 1 hr under 95% O_2 and 5% CO_2 in a shaking bath (120 cycles/min). After preincubation, the cells were collected by centrifugation (100*g*, 5 min), washed once with incubation medium, and suspended in 1 ml incubation medium containing or lacking either or both LH (2.5–80 ng/ml/tube; ovine LH: NIDDK oLH-S26) or melatonin (0.5, 1 and 5 n*M*). The concentrations of melatonin solutions containing 0.05% ethanol were prepared from a stock solution (5 mg/ml ethanol) by diluting with incubation medium to a desired concentration. The tubes were then incubated again at 40.5°C for 4 hr. After the incubation, the tubes were immediately cooled in an ice bath, and were centrifuged (1000*g*, 10 min, 4°C). The supernatants were stored at −20°C until progesterone (P_4) assay. The P_4 concentration was measured by routine RIA (36). In preliminary experiments, time course of P_4 production by LH (80 ng/tube) during 1–6 hr of incubation was examined. It was found that the amount of P_4 produced increased during the first 3 hr of incubation, and then reached a plateau. On the basis of this finding, a 4-hr incubation was used in experiments.

Statistical Analyses. Kinetic data were analyzed by the method of Bylund and Yamamura (37), 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}). A half-maximal response (ED_{50}) was estimated by the use of a log-probit linear regression (38, 39). For comparisons among more than two groups, the data were analyzed by one-way analysis of variance (ANOVA) (40). When significant ($P < 0.05$) effects were found, Tukey's multiple range test was used to separate means (40).

Results

Relationship of Specific [^{3}H]melatonin Binding to Incubation Period. The specific [^{3}H]melatonin binding in the granulosa of hens increased during the first 30 min of incubation and was then maintained up to 120 min (Fig. 1). In the theca, specific binding was not demonstrated up to 120 min of incubation (Fig. 1).

Relationship of Specific [^{3}H]melatonin Binding to Protein Concentration of Membrane Fraction. Specific [^{3}H]melatonin binding in the granulosa of hens increased linearly with increase in the protein concentration from 0.1 to 0.8 mg/tube when incubated for 60 min (Fig. 2). In contrast, specific binding in the theca did not increase with the increase in the protein concentration up to 0.8 mg/tube (Fig. 2).

Binding Affinity and Capacity. Specific [^{3}H]melatonin binding in the membrane fraction of granulosa of hens increased when increasing amounts of [^{3}H]melatonin were added (i.e., when the amount of free [^{3}H]melatonin was increased), and was saturable at about 1.2 n*M* (Fig. 3). Scathchard analysis revealed a linear relationship between the amount of specific [^{3}H]melatonin binding and the ratio (B/F) of specific [^{3}H]melatonin binding to free [^{3}H]melatonin (Fig. 3), indicating a single class of binding sites. The value of K_d and B_{max}, and correlation coefficient (r) between B/F and specific [^{3}H]melatonin binding obtained from four separate pools of samples were 0.67 ± 0.08 n*M* (K_d), 3.27 ± 0.41 fmol/mg protein (B_{max}), and −0.95 to −0.98 (r), respectively.

Kinetic Analysis of [^{3}H]melatonin Binding. When [^{3}H]melatonin was used at a concentration equivalent to the K_d value (0.7 n*M*) for the incubation of the same four separate pools of samples as used for the measurement of binding affinity and capacity, the specific [^{3}H]melatonin binding in the membrane fraction of granulosa of hens reached a steady state at 30 min and was stable for up to 120 min (Fig. 4). The addition of a large excess of unlabeled melatonin caused a decrease in specific [^{3}H]melatonin binding. The association rate constant (K_{+1}) was 0.0701 ± 0.0010 nM^{-1} min^{-1}. Specific [^{3}H]melatonin binding was reduced ($t_{1/2}$ = 15.2 ± 0.5 min) by the addition of a large excess of unlabeled melatonin. The rate constant for dissociation (K_{-1}) determined from the pseudo-first-order equation was 0.0475 ± 0.0017 min^{-1}. The kinetic dissociation constant (K_d) for [^{3}H]melatonin calculated from the ratio K_{-1}/K_{+1} was 0.68 ± 0.03 n*M*.

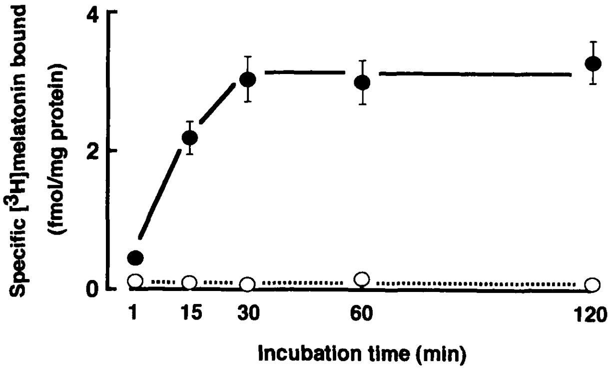

Figure 1. Time course of specific [^{3}H]melatonin binding in membrane fractions of the granulosa (●) and theca (○) cells of hens. Samples (350 µg protein/tube) were incubated at 4°C for various times (min) with 0.7 n*M* [^{3}H]melatonin in the absence or presence of a 1000-fold molar excess (0.7 µ*M*) of unlabeled melatonin. Each point represents the mean ± SEM of three separate pools of samples. Standard errors not shown fall within the point as drawn.

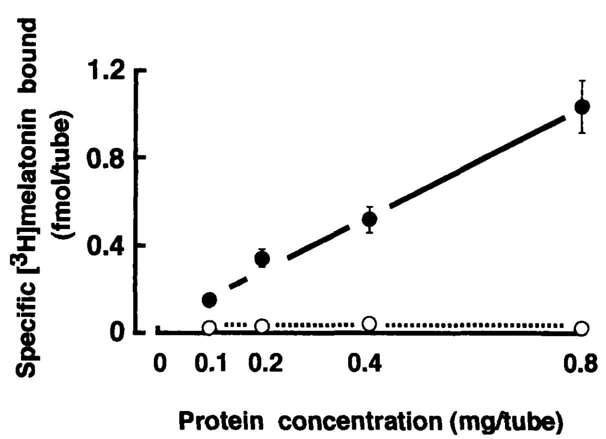

Figure 2. Relationship of specific [^{3}H]melatonin binding to protein concentration in membrane fractions of the granulosa (●) and theca (○) cells of hens. Samples (0.1–0.8 mg protein/tube) were incubated at 4°C for 60 min with 0.7 n*M* [^{3}H]melatonin in the absence or presence of a 1000-fold molar excess (0.7 µ*M*) of unlabeled melatonin. Each point represents the mean ± SEM of three separate pools of samples. Standard errors not shown fall within the point as drawn.

Binding Specificity. The [^{3}H]melatonin binding at a concentration of 0.7 n*M* (equivalent to the K_d value) in the membrane fraction of granulosa of hens was markedly reduced by the presence of a 100-fold molar excess of unlabeled melatonin (70 n*M*), but little affected by the presence of an equivalent molar concentration of unlabeled 6-hydroxymelatonin, 5-hydroxytryptophan, serotonin, *N*-acetylserotonin, 5-methoxytryptophol, or 5-methoxyindole acetic acid (Fig. 5). These melatonin-related substances reduced the binding (28% to 47%) when a 10,000-fold molar excess was used.

Effect of Melatonin on Granulosa Cell Responsiveness to LH. Figure 6 shows the dose-response relationship of LH-stimulated progesterone production in the granulosa cells of the hen. The dose-response curve of melatonin-treated cells was shifted to a higher concentration of LH. The ED_{50} value for LH was greater with treatments of 1 or 5 n*M* melatonin than in the treatments of vehicle and 0.5 n*M* melatonin (Table I). However, the maximal amount of LH-stimulated progesterone was not significantly different among the groups (Table I).

Discussion

The membrane fraction of granulosa cells of the hen was found to contain a melatonin-binding component possessing a high affinity and a limited capacity (Fig. 3). The binding was reversible (Fig. 4) and specific (Fig. 5). These properties are characteristics of a receptor, and therefore, the presence of a melatonin receptor in the hen granulosa cells is suggested.

The K_d value (0.68 ± 0.03 n*M*) calculated from kinetic analysis is in close agreement with that (0.67 ± 0.08 n*M*)

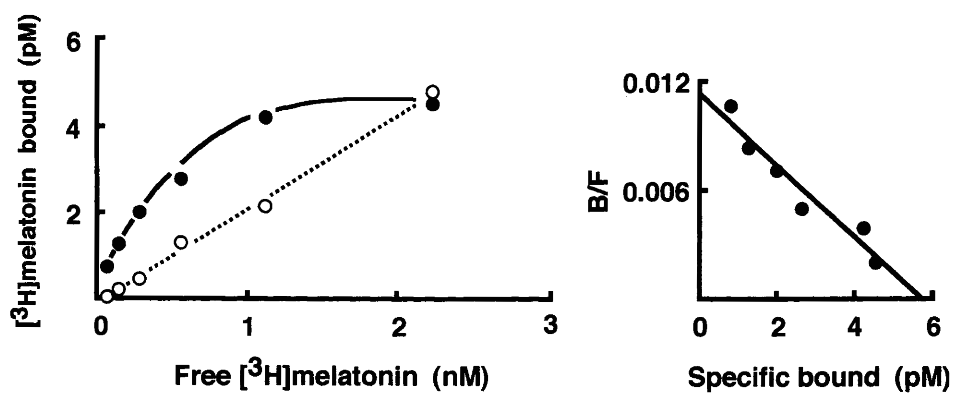

Figure 3. Saturation curves and Scatchard plots of specific [^{3}H]melatonin binding in membrane fraction of the granulosa cells of hens. Samples (350 µg protein/tube) were incubated at 4°C for 60 min with various concentrations (0.08–2.6 n*M*) of [^{3}H]melatonin in the absence or presence of a 1000-fold molar excess of unlabeled melatonin. The value of K_d and B_{max} (calculated by the use of Scatchard analysis) and correlation coefficient (r) between B/F and specific binding was 0.51 n*M* (K_d), 3.2 fmol/mg protein (B_{max}) and –0.96 (r), respectively. Each point represents the mean of duplicate determinations from one pooled sample. ●, specific binding; ○, nonspecific binding; B, specific [^{3}H]melatonin bound; F, free [^{3}H]melatonin.

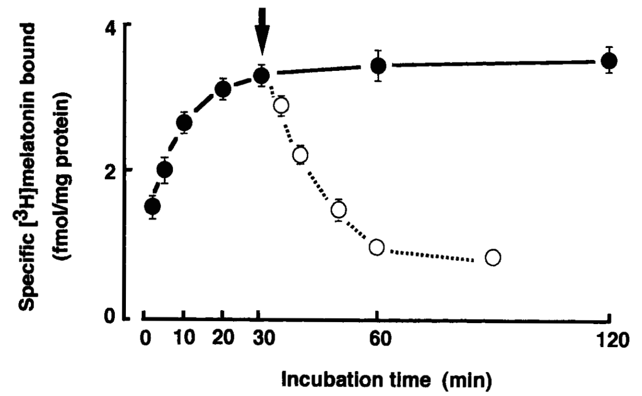

Figure 4. Association (●) and dissociation (○) of [^{3}H]melatonin binding in membrane fraction of the granulosa cells of hens. Samples (350 µg protein/tube) were incubated at 4°C with 0.7 n*M* of [^{3}H]melatonin in the absence or presence of a 1000-fold molar excess (0.7 µ*M*) of unlabeled melatonin. The arrow indicates the addition of 7 µ*M* (0.34 µg/10 µl assay buffer) of unlabeled melatonin. Each point represents the mean ± SEM of four separate pools of samples. Standard errors not shown fall within the point as drawn.

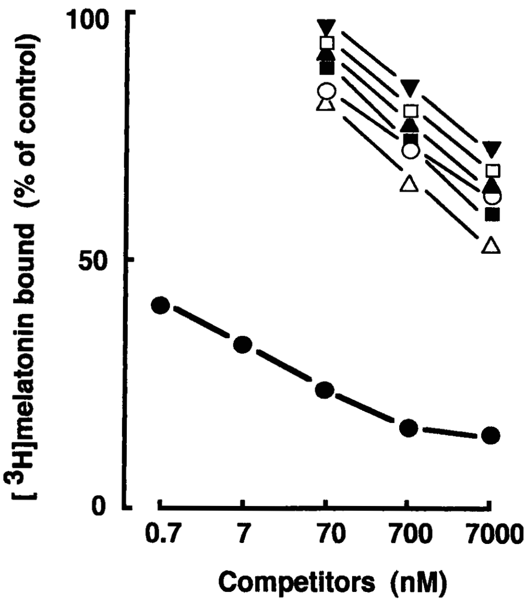

Figure 5. Competition for [^{3}H]melatonin binding in membrane fractions of the granulosa cells of hen. Samples (350 µg protein/tube) were incubated at 4°C for 60 min with 0.7 n*M* [^{3}H]melatonin in the absence (control) or presence of various-fold molar excesses of unlabeled competitors. The amount of [^{3}H]melatonin binding in the control value was 1.95 fmol/mg protein. Each point represents the mean of two separate pools of samples. The competitors tested were: melatonin (●), 6-hydroxymelatonin (△), 5-hydroxytryptophan (▼), serotonin (○), *N*-acetylserotonin (■), 5-methoxytryptophol (□), 5-methoxyindole acetic acid (▲).

obtained by Scatchard analysis of saturation studies. That the binding sites are of a single class is consistent with the finding on human granulosa cells (17), the duck ovary (16), and the testis of quail (18, 19) and rats (20). The K_d value (indicative of the degree of binding affinity) expressed as moles per liter was of the order of 10^{-10} in the present study. A similar order of K_d value was reported on the retina (0.13 n*M*) of frogs (41), and the hypothalamus (0.17 n*M*) (42), harderian gland (0.19 n*M*) (42), testis (0.10 n*M*) (20), thymus (0.47 n*M*) (43), and splenocytes (0.34 n*M*) (44) of rats. All these values were obtained by the use of the radioiodinated melatonin analog, 2-[^{125}I]iodomelatonin (MW = 356). A different order of the K_d value obtained by the use of the analog was also reported on central and peripheral tissues of various species of animals: the avian brain (22–92 p*M*) (45, 46), kidney (30–45 p*M*) (47–49), lung (9 p*M*) (50), heart (10–30 p*M*) (50), bursa of fabricius (28–73 p*M*) (51), thymus (30–35 p*M*) (52), spleen (37–47 p*M*) (53), liver (19–25 p*M*) (54), gastrointestinal tract (41 p*M*) (55), spinal cord (12–15 p*M*) (56), testis (28–35 p*M*) (16, 19), and ovary (53 p*M*) (16), and the mammalian hypothalamus (29–39 p*M*) (57), pars tuberalis (21 p*M*) (6), pars distalis (81 p*M*) (6), spleen (42–50 p*M*) (58), kidney (15–22 p*M*) (59, 60), and granulosa cells (99 p*M*) (17). These values are at an extremely higher level considering the level of the endogenous melatonin secretion, which is known to change during a daily light:dark cycle (61). The plasma concentration of melatonin has been reported to be 340 p*M* (light) and

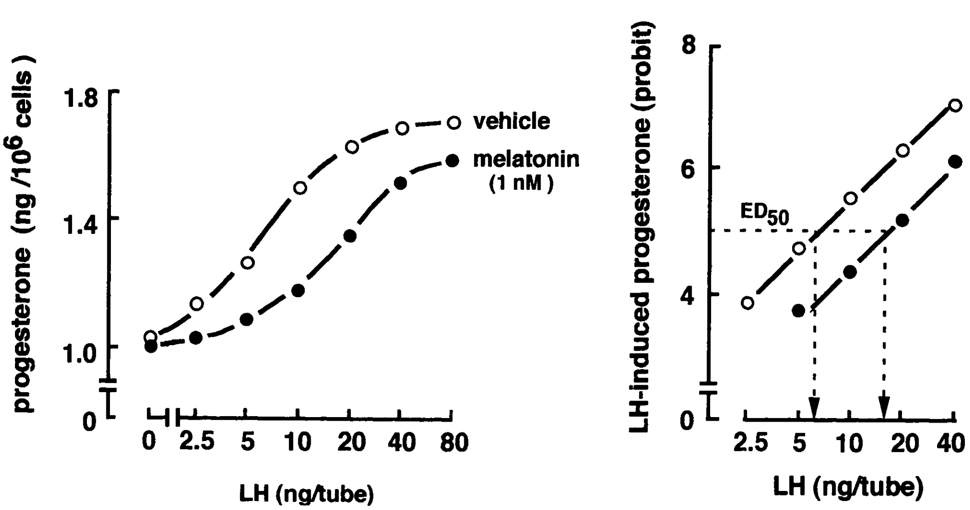

Figure 6. Effects of melatonin treatment on progesterone production by LH in isolated granulosa cells of hen. The isolated cells (1 × 10^6 cells/tube) were incubated at 40.5°C for 4 hr with the incubation medium containing or not containing either or both LH (various concentrations) and melatonin (1 n*M*). The panels show the dose-response relationship for LH with or without melatonin treatment (left), and the relationship between LH concentrations and probit values (right) that was calculated from data of the left panel. The amount of progesterone in the control (without both hormones) was 1.03 ng/ml/tube. The half-maximal response (ED_{50}) values for LH calculated from the data of dose-response curves were 6.7 ng/ml for nontreatment, and 15.8 ng/ml for melatonin treatment, respectively. Each point represents the mean of two tubes in one incubation. Broken line with arrow, ED_{50}.

Table I. Half-Maximal Response (ED_{50}) and Maximal (Max) Amount of LH-Stimulated Progesterone (P_4) following the Incubation of Ovarian Granulosa Cells of the Hen in Medium Containing or Not Containing Either or Both LH (2.5–80 ng/Tube) and Melatonin

Melatonin (n*M*)	ED_{50} (ng LH/10^6 cells)	Max P_4 (ng/10^6 cells)
Vehicle	6.8 ± 0.5^a	1.72 ± 0.07^a
0.5	8.1 ± 0.6^a	1.66 ± 0.11^a
1	16.1 ± 1.0^b	1.60 ± 0.10^a
5	13.2 ± 0.7^b	1.78 ± 0.14^a

Note. The cells (1 × 10^6/tube) were incubated at 40.5°C for 4 hr. P_4 in the incubation medium was measured by routine RIA. ED_{50} for the LH-stimulated P_4 production was estimated by the use of a log-probit linear regression (Fig. 6). The amount of P_4 in the control lacking both LH and melatonin was 0.95 ± 0.07 (mean ± SEM; $n = 4$) ng/10^6 cells/tube. Values are mean ± SEM of four separate pools of samples.

[a,b] Means in the same column with no common superscripts are significantly different ($P < 0.01$) by Tukey's multiple range test.

1700–2400 p*M* (dark) in the chicken (62), 214 p*M* (mid-light) and 670 p*M* (mid-dark) in the duck (47), and 152 p*M* (mid-light) and 562 p*M* (mid-dark) in the pigeon (63). Even the lower value of the blood concentration during the light phase is higher than the values of the binding affinity of receptor reported by the use of the radioiodinated melatonin analog. This means that melatonin is always acting on the target tissues irrespective of day-night rhythms of its secretion. If endogenous melatonin rhythms are concerned in its action on target tissues, the extremely high affinity receptor may not be of physiological significance. Therefore, the binding affinity (K_d value) obtained in the present study may represent an appropriate value reflecting the diurnal change in the melatonin concentration.

The B_{max} value (3.27 ± 0.41 fmol/mg protein) obtained in the present study is of a similar order of value to that reported on various melatonin target tissues of birds (16, 18, 19, 45, 46, 48–52, 54–56) and mammals (6, 17, 20, 57–60).

When the granulosa cells were incubated *in vitro,* the dose-response curves for the LH-stimulated progesterone (P_4) production shifted to a higher concentration of LH (Fig. 6)—that is, the half-maximal response (ED_{50}) was greater in the melatonin-treated cells (Table I)—indicating that the responsiveness of the granulosa cells to LH is decreased by melatonin. The effect may be due to a direct action of melatonin on the granulosa cells, because the effect was apparent in the melatonin concentrations (1 and 5 n*M*) above the value of K_d (0.67 or 0.68 n*M*) of receptor, but not in the concentration (0.5 n*M*) of below the K_d value.

The results of the present study suggest that one of the actions of melatonin on the granulosa cells of the hen is to lower the responsiveness to LH for the production of progesterone through binding to its receptors existing in the cells.

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