

# PHYSIOLOGY AND REPRODUCTION

## Mesotocin Receptor Binding of Cortical and Medullary Kidney Tissues of the Hen

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**ABSTRACT** [<sup>125</sup>I]Mesotocin (MT) binding of membrane fractions of the cortical tissue and the medullary tissue (medullary cone) of the kidney of nonlaying hens was measured by the use of radioligand binding assays to determine the distribution of two distinct MT receptors within the hen kidney. The binding to [<sup>125</sup>I]MT in the medullary tissue was found to be highly competitive with unlabeled MT. In the cortical tissue, the binding was competitive with both unlabeled MT

and arginine vasotocin. Kinetic and Scatchard analyses of specific binding revealed that the binding affinity was higher in the cortical tissue than in the medullary tissue, but the binding capacity was less in the cortical tissue. The localization of two distinct MT receptor having different binding properties may be related to the biphasic action of MT within the tissue of the kidney of the hen.

(Key words: nonlaying hen, kidney cortical tissue, kidney medullary tissue, mesotocin receptor, mesotocin binding)

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### INTRODUCTION

Mesotocin (MT) is a neurohypophysial hormone in birds (Acher *et al.*, 1970). When injected into the hen, it causes antidiuresis or diuresis depending on the dose (Takahashi *et al.*, 1995). The existence of two distinct receptors for MT having a higher and a lower binding affinity was recently demonstrated in the membrane fraction of the kidney of the hen by radioligand binding assays (Takahashi *et al.*, 1996). The hen kidney consists of three divisions without histological difference (Morild *et al.*, 1985). The kidney consists of many lobules. A lobule has a cortical region and a medullary region. The medullary region of a lobule is also known as a medullary cone (Siller, 1981; Wideman, 1988; King, 1993). When dissected, cortical and medullary regions of lobules are visually recognized easily in all of the divisions by a difference of their colors. The present study was performed to determine the distribution of two distinct MT receptors within the hen kidney.

### MATERIALS AND METHODS

#### Animals and Tissues

White Leghorn hens (20 mo of age; 1.6 to 1.8 kg body weight) were kept under 14 h light (0500 to 1900 h)/d with feed (15% CP; 2,800 kcal ME; Japan Feeding Standard for Poultry, 1992) and water provided for *ad libitum* consumption. To avoid the possible effect of endogenous secretion of neurohypophysial hormones in relation to oviposition, only hens that had not laid an egg for at least 10 d prior to experiments were used. The hens were killed by decapitation at 1000 h, and whole divisions of kidneys of both sides were excised and rinsed with ice-cold Tris-EDTA buffer (TE; 50 mM Tris<sup>2</sup>-HCl, 2 mM EDTA,<sup>3</sup> pH 7.4) containing 0.25 M sucrose.<sup>3</sup> For each experiment, one bird was used. The divisions of the kidney were cut frontally into slices of approximately 2 mm thickness by the use of a surgical blade. From all of the slices, cortical tissues with a width of approximately 1 mm and medullary tissues of approximately 5 mm in diameter were excised under a binocular microscope and pooled (total 2.4 to 3.3 g of cortical tissue per bird; 2.5 to 3.4 g of medullary tissue per bird). The pooled tissues were immediately used for the preparation of membrane fractions.

#### Preparation of Membrane Fractions

The sucrose gradient centrifugation method used for the preparation of membrane fractions was the same as

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reported earlier (Takahashi *et al.*, 1996). The kidney tissues were homogenized in TE buffer containing 0.25 M sucrose, and centrifuged (800 × g, 10 min, 4 C). The precipitate was rehomogenized and recentrifuged. The supernatants were combined and centrifuged (30,000 × g, 30 min, 4 C), and the supernatant was suspended in the same buffer. The suspension was gently poured on TE buffer containing 1.0 M sucrose. After centrifugation (90,000 × g, 90 min, 4 C) in a swinging rotor (RPS 65T),<sup>4</sup> the interface fraction was collected. The fraction was washed twice with TE buffer without sucrose by centrifugation (30,000 × g, 30 min, 4 C). The precipitate was suspended in the same buffer and used as the membrane fraction after measuring the protein concentration by the method of Lowry *et al.* (1951) using BSA (Fraction V)<sup>5</sup> as a standard. The amount of protein in the membrane fraction was 1.21 ± 0.02 ( $\bar{x}$  ± SEM, n = 5) μg/g cortical tissue and 1.28 ± 0.03 μg/g medullary tissue, respectively.

### Mesotocin Binding Assay

The labeling of MT with <sup>125</sup>I was performed by the Iodogen method (Takahashi *et al.*, 1992). Specific activity of the [<sup>125</sup>I]MT determined by the method of Copeland *et al.* (1979) was 1,896 to 2,471 Ci/mmol. The membrane fractions (5 μg protein per tube), to which [<sup>125</sup>I]MT (0.03 to 2.4 nM) was added, were incubated at 30 C for 1 h (cortical tissue) or 5 h (medullary tissue) in the presence (for nonspecific binding) or absence (for total binding) of 1 μM of unlabeled MT as a total volume of 300 μL. For the examination of reversibility of [<sup>125</sup>I]MT binding, unlabeled MT (1 μM) was added at 30 min (cortical) or 4 h (medullary) of incubation, and the incubation was continued up to 4 h (cortical) or 10 h (medullary). For experiments on competitive binding, 7 nM to 7,000 nM of unlabeled MT,<sup>6</sup> oxytocin (OT),<sup>6</sup> AVT,<sup>6</sup> and arginine vasopressin (AVP),<sup>6</sup> and 70 nM to 7,000 nM of unlabeled chicken (c) luteinizing hormone-releasing hormone-I (cLHRH-I: Gln<sup>8</sup>-GnRH),<sup>7</sup> cLHRH-II (His<sup>5</sup>, Tyr<sup>7</sup>, Tyr<sup>8</sup>-GnRH),<sup>6</sup> and cAngiotensin-II (Val<sup>5</sup>-angiotensin-II)<sup>6</sup> were used. Separation of bound and free ligands were performed by centrifugation (10,000 × g, 20 min, 4 C), and the precipitate was washed with TE buffer by the same centrifugation. The radioactivity of bound ligands in the precipitate was measured by a gamma counter (Packard Cobra).<sup>8</sup> The counting efficiency for <sup>125</sup>I was 66 to 88%. The specific binding was obtained by subtracting the nonspecific binding from the total binding and expressed as moles per milligram of protein. The equilibrium dissociation constant (K<sub>d</sub>) and the maximum binding capacity (B<sub>max</sub>) were determined by the method of Scatchard (1949).

### Statistical Analyses

Kinetic data were analyzed by the use of pseudo-first-order conditions (Bylund and Yamamura, 1990) to estimate the association rate constant (K<sub>+1</sub>) and the dissociation rate constant (K<sub>-1</sub>) after the addition of a large excess of unlabeled ligand. Student's *t* test (Snedecor and Cochran, 1967) was used to assess the statistical significance of difference between two means when significant (*P* < 0.05) effects were found by one-way ANOVA.

## RESULTS

### Kinetic Analysis of [<sup>125</sup>I]Mesotocin Binding

In the membrane fractions of the cortical tissue of the hen kidney, the specific binding of [<sup>125</sup>I]MT reached a steady state at 30 min of incubation and was stable for up to 240 min. In the membrane fraction of the medullary tissue, the specific binding reached a steady state at 240 min and was stable for up to 600 min (Figure 1). A decrease in the specific [<sup>125</sup>I]MT binding occurred upon adding a large excess of unlabeled MT (Figure 1). The binding of [<sup>125</sup>I]MT in the membrane fraction of the cortical and medullary tissues was reversible and time dependent. The association rate constant (K<sub>+1</sub>) was 0.223 ± 0.022 nM<sup>-1</sup> min<sup>-1</sup> ( $\bar{x}$  ± SEM; n = 5) in the cortical tissue and 0.0081 ± 0.0006 nM<sup>-1</sup> min<sup>-1</sup> (n = 5) in the medullary tissue, respectively. Specific [<sup>125</sup>I]MT binding was reversed by the addition of unlabeled MT [half-time (t<sub>1/2</sub>) = 4.7 ± 0.8 min (n = 5) in cortical tissue and t<sub>1/2</sub> = 71.0 ± 9.3 min (n = 5) in medullary tissue]. The rate constant for dissociation (K<sub>-1</sub>) determined from the pseudo-first-order equation was 0.018 ± 0.001 min<sup>-1</sup> in the cortical tissue and 0.0064 ± 0.0001 min<sup>-1</sup> in the medullary tissue. The kinetic dissociation constant (K<sub>d</sub>) for [<sup>125</sup>I]MT binding, calculated from the ratio, K<sub>-1</sub>:K<sub>+1</sub> was 0.083 ± 0.007 nM (n = 5) in the cortical tissue and 0.80 ± 0.05 nM (n = 5) in the medullary tissue, respectively. The difference between the K<sub>d</sub> values of the cortical and the medullary tissues was statistically significant at 1% level.

### Binding Specificity

The [<sup>125</sup>I]MT binding in the medullary tissue was markedly reduced by the presence of unlabeled MT. A 10-fold molar excess concentration of the unlabeled MT reduced the binding about 50%. In the presence of AVT, the reduction of binding to approximately 50% was observed at the concentration of a 10,000-fold molar excess. The mammalian neurohypophysial hormones, OT and AVP, reduced the binding to a less extent than that of MT (Figure 2). In the cortical tissue, both MT and AVT reduced the binding. In both tissues, the presence of cLHRH-I, cLHRH-II, and cAngiotensin-II did not reduce the [<sup>125</sup>I]MT binding.

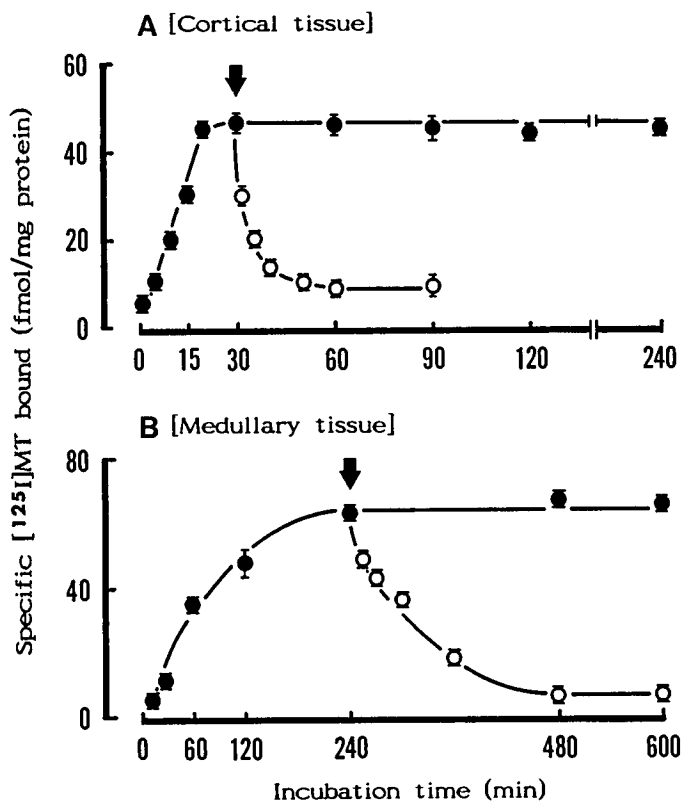
<sup>4</sup>Hitachi Koki Co., Ltd., Hitachinaka 312, Japan.

<sup>5</sup>Seikagaku Corp., Tokyo 103, Japan.

<sup>6</sup>Bachem Inc., Torrance, CA 90505.

<sup>7</sup>Peninsula Laboratories, Inc., Belmont, CA 94002.

<sup>8</sup>Packard Instrument Co., Meriden, CT 06450.



**FIGURE 1.** Time-course of the association (●) and dissociation (○) of [<sup>125</sup>I]mesotocin (MT) in plasma membrane fractions of cortical (A) and medullary (B) tissues of the kidney of the hen. Samples (5 μg protein per tube) were incubated at 30 C for various times with 0.7 nM [<sup>125</sup>I]MT in absence or presence of 1 μM unlabeled MT, and specific [<sup>125</sup>I]MT bindings were measured. Specific [<sup>125</sup>I]MT binding decreased following the addition of 1 μM unlabeled MT (arrow). Each point represents the mean ± SEM of five separate pools of samples.

### Binding Affinity and Capacity

The specific [<sup>125</sup>I]MT binding was increased with the increase in the amount of [<sup>125</sup>I]MT in both cortical and medullary tissues, and reached a plateau at about 0.3 nM in the cortical tissue and at about 1.2 nM in the medullary tissue (Figure 3). Scatchard plots revealed a linear relationship between the amount of specific [<sup>125</sup>I]MT binding and the ratio (B:F) of the specific binding to the amount of free [<sup>125</sup>I]MT in both tissues (Figure 3),

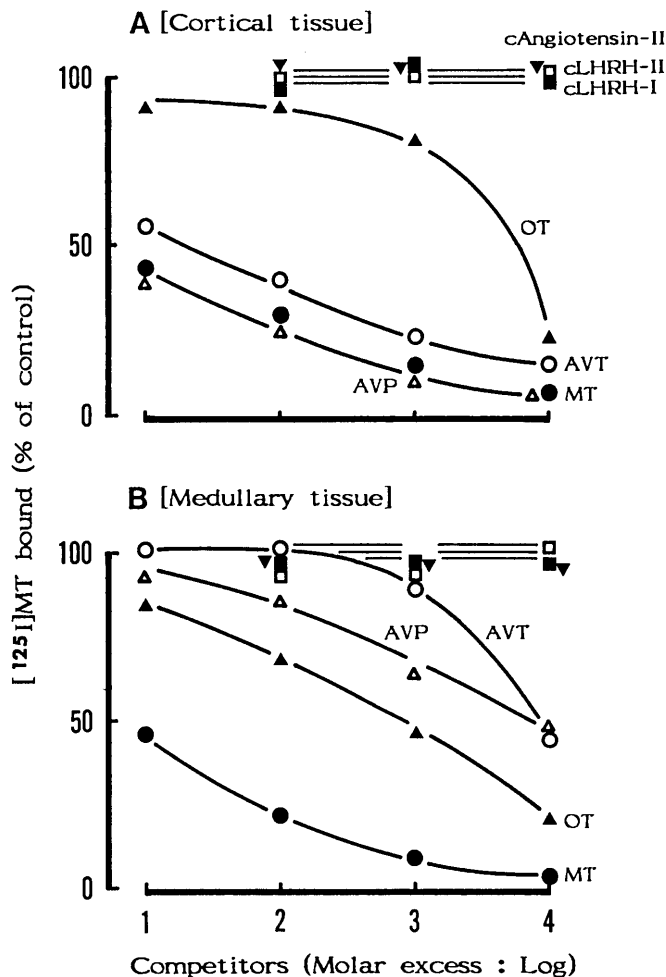
**TABLE 1.** Equilibrium dissociation constant ( $K_d$ ) and maximum binding capacity ( $B_{max}$ ) of mesotocin (MT) receptors in plasma membrane fractions of cortical and medullary tissues of the kidney in hens<sup>1</sup>

Tissue	$K_d$ (nM)	$B_{max}$ (fmol/mg protein)
Cortical tissue	0.081 ± 0.002 <sup>2</sup> **	46 ± 1 **
Medullary tissue	0.76 ± 0.02	141 ± 3

<sup>1</sup>Calculated by the use of Scatchard (1949) analysis.

<sup>2</sup>Mean ± SEM of five separate pools of samples.

\*\* $P < 0.01$ .

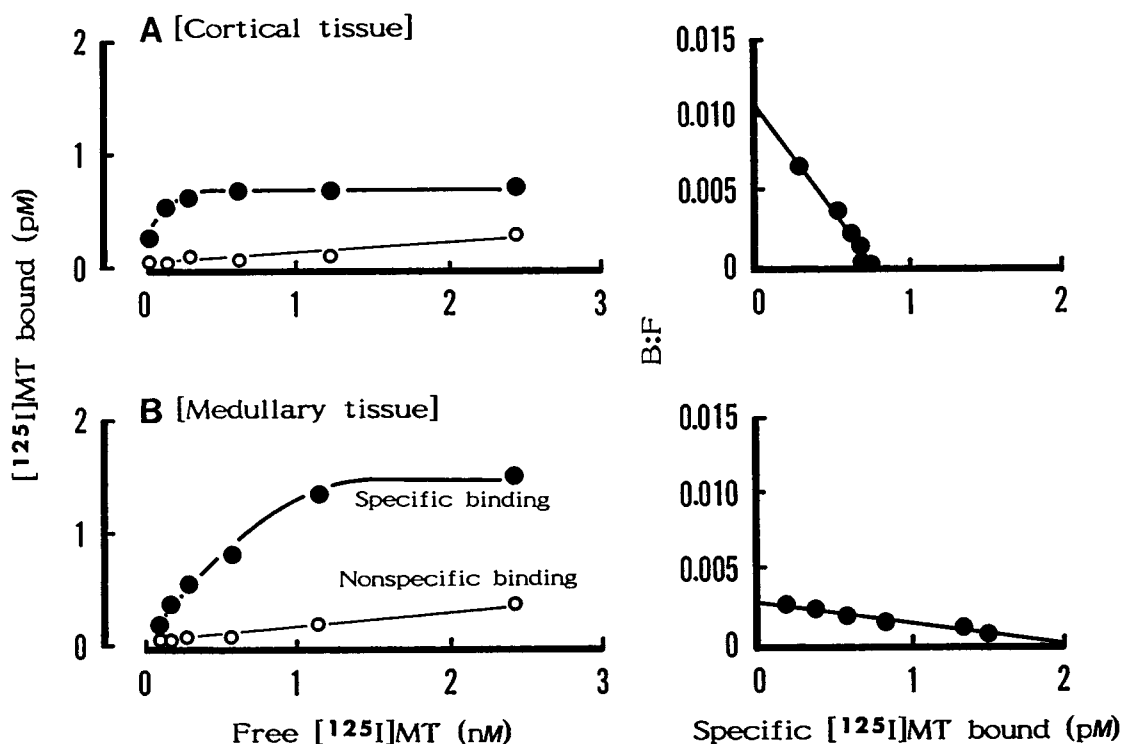


**FIGURE 2.** Competition for [<sup>125</sup>I]mesotocin (MT) bindings in plasma membrane fractions of cortical (A) and medullary (B) tissues of the kidney of the hen. Samples (5 μg protein per tube) were incubated at 30 C for 1 h (cortical) or 5 h (medullary) with 0.7 nM [<sup>125</sup>I]MT in the absence (control) or presence of various fold molar excess (1 = 7 nM; 2 = 70 nM; 3 = 700 nM; 4 = 7,000 nM) of unlabeled competitors. In control value, amounts of [<sup>125</sup>I]MT binding were 53 fmol/mg protein in the cortical tissue and 71 fmol/mg protein in the medullary tissue. Each point represents the mean of two separate pools of samples. The competitors tested were: MT (●), arginine vasotocin (AVT; ○), oxytocin (OT; ▲), arginine vasopressin (AVP; △), chicken luteinizing hormone-releasing hormone-I (cLHRH-I; ■), cLHRH-II (□), and chicken angiotensin-II (cAngiotensin-II; ▼).

indicating one single class of binding sites in each tissue. Table 1 shows the  $K_d$  and  $B_{max}$  values obtained by the Scatchard analysis of the specific [<sup>125</sup>I]MT binding in the cortical and the medullary tissues of the kidney. The difference between the  $K_d$  values of the cortical and the medullary tissues was statistically significant ( $P < 0.01$ ). For the  $B_{max}$  values, the difference between the two tissues was also statistically significant ( $P < 0.01$ ).

### DISCUSSION

The present work corroborated previous research (Takahashi *et al.*, 1996) in which distinct MT receptors have been identified in the hen's kidney, and corroborated the distribution of the two distinct MT receptors



**FIGURE 3.** Saturation curve and Scatchard plot of specific bindings for [ $^{125}$ I]mesotocin (MT) in plasma membrane fractions of cortical and medullary tissues of the kidney of the hen. Samples ( $5 \mu\text{g}$  protein per tube) were incubated at  $30^\circ\text{C}$  for 1 h (cortical) or 5 h (medullary) with various concentrations of [ $^{125}$ I]MT in the absence or presence of  $1 \mu\text{M}$  unlabeled MT, and specific [ $^{125}$ I]MT bindings were measured. Values of equilibrium dissociation constant ( $K_D$ ) were  $0.072 \text{ nM}$  in the cortical tissue and  $0.69 \text{ nM}$  in the medullary tissue, maximum binding capacity ( $B_{\text{max}}$ ) values were  $45 \text{ fmol/mg}$  protein in the cortical tissue and  $120 \text{ fmol/mg}$  protein in the medullary tissue, and correlation coefficient ( $\gamma$ ) between B:F and specific binding were  $-0.994$  in the cortical tissue and  $-0.970$  in the medullary tissue. Each point represents the mean of duplicate determinations from one pooled sample. (●) Specific binding, (○) Nonspecific binding. B = bound; F = free.

within the hen kidney. The higher affinity receptor was found in the cortical tissue and the lower affinity receptor was in the medullary tissue. Although there is a possibility that either tissue contains two types of MT receptors within that tissues, high and low affinity MT receptor may be contained mainly in the cortical tissue and the medullary tissue, respectively. Because the results of the Scatchard plot revealed linearity (Figure 3), the two receptors were apparently different not only in the binding affinity and capacity but also in the competition for the binding to [ $^{125}$ I]MT with other neurohypophysial hormones such as OT, AVP, and AVT (Figure 2). The difference in the binding properties suggest a difference in the physiological role of MT receptors in the kidney of the hen. Recent findings on the effect of exogenous MT on the excretion of urine in the hen indicate that MT exerts either antidiuretic or diuretic action depending on the dose: a lower dose of MT ( $0.0025$  to  $0.25 \mu\text{g/kg}$  body weight) was injected into hens, the volume of urine was decreased. However, when a higher dose ( $2.5$  to  $25 \mu\text{g/kg}$  body weight) was injected, the urine volume was increased. The existence of two receptors having different binding properties may be related to the biphasic action of MT. According to histological observations on the avian kidney (Braun and Dantzler, 1972; Braun, 1976; Siller,

1981; Braun, 1982; Wideman, 1988; King, 1993), the cortical tissue contains glomeruli and tubules of nephrons, and the medullary tissue (medullary cones) contains mainly tubules as loops of Henle. In the mammalian kidney, receptors for AVP have been reported to be present in tubules of nephrons (Dorsa *et al.*, 1983), and in the amphibian kidney, receptors for AVT in glomeruli of nephrons (Boyd and Moore, 1990). Although no histological observations for the localization of MT receptors in the kidney structures were made in the present study, the presence of two distinct MT receptors found in the membrane fractions of the kidney tissue of the hen, one in the cortical tissue and the other in the medullary tissue, suggest the localization of MT receptors in either the glomeruli or tubules of nephrons influencing water-balance in the hen.

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