PHYSIOLOGY AND REPRODUCTION

Mesotocin Binding to Receptors in Hen Kidney Plasma Membranes

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ABSTRACT Radioligand assays were performed to demonstrate the presence of a receptor for mesotocin (MT) in the membrane fractions of the kidney of the hen. Specific [^{125}I]MT bindings were decreased by the presence of Mg^{2+} and Ca^{2+}, increased by the presence of EDTA, increased during the first 4 h of incubation and then reached a plateau, and increased with the increase in the protein concentration from 2.5 to 20 μg. The membrane fraction showed binding specificity to [^{125}I]MT. The Scatchard plot revealed a curvilinear profile that indicated the presence of two classes of binding sites: a high affinity site and a low affinity site. The equilibrium dissociation constant was 0.08 ± 0.01 nM ($\bar{x}$ ± SEM; n = 5) in the high affinity site and 0.87 ± 0.08 nM (n = 5) in the low affinity site. The maximum binding capacity of the high and low affinity sites was 42 ± 4 and 129 ± 6 fmol/mg protein, respectively. The results suggest the presence of two distinct MT receptors in the kidney of the hen.

(*Key words*: hen, kidney, mesotocin, mesotocin binding, mesotocin receptor)

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INTRODUCTION

Mesotocin (MT), one of the two neurohypophysial hormones in birds (Acher *et al.*, 1970), is a nona-peptide having a similar chemical structure to the other neurohypophysial hormone, arginine vasotocin (AVT). The only difference between the chemical structures of the two neurohypophysial hormones is the difference in the amino acid residue at the position 8: for MT it is isoleucine and for AVT it is arginine (Sawyer, 1977; Acher, 1985). Arginine vasotocin is known as an antidiuretic hormone in birds (Skadhauge, 1964; Ames *et al.*, 1971; Bradley *et al.*, 1971; Braun and Dantzler, 1974), causing an increase in the reabsorption of water from the renal tubules (Nishimura *et al.*, 1984) as in mammals (Valtin, 1966; Hai and Thomas, 1969; Atherton *et al.*, 1971); but the physiological role of MT is obscure. The chicken plasma concentration of MT increases following dehydration (Nouwen *et al.*, 1984) or infusion of hypotonic saline solution (Koike *et al.*, 1986). When MT is injected into the hen *in vivo*, antidiuretic or diuretic effects are manifested depending on the dose injected (Takahashi *et al.*, 1995). Whether the two reciprocal effects of MT on the excretion of urine are due to a direct action of MT on the kidney is unknown. The present study was undertaken to demonstrate the presence of a receptor for MT in the renal tissue of the hen.

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. Only hens that had not laid an egg for at least 10 d prior to experiments were killed by decapitation at 1000 h. Both kidneys were excised, rinsed with ice-cold Tris-EDTA buffer (TE; 50 mM Tris[2]-HCl, 2 mM EDTA,[3] pH 7.4) containing 0.25 M sucrose,[3] blotted with a filter paper, and used immediately for the preparation of membrane fraction.

Preparation of Membranes

The methods used were the same as reported earlier (Takahashi *et al.*, 1992). All procedures for the preparation of membrane fraction were carried out at 4 C. The kidneys were homogenized in TE buffer containing 0.25 M sucrose and centrifuged at 800 × g for 10 min. The precipitate was homogenized again and centrifuged. The supernatants were pooled and centrifuged at 30,000 × g for 30 min, and

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the precipitate was resuspended in the same buffer. The suspension was gently poured on the surface of TE buffer containing 1.0 *M* sucrose and centrifuged at 90,000 × *g* for 90 min in a swinging rotor (RPS 65T).[4] The interface fraction was collected and washed twice with TE buffer, not containing sucrose, by centrifugation (30,000 × *g*, 30 min). The precipitate was suspended in the same buffer, and was stored at –80 C as a membrane fraction. The protein concentration was measured by the method of Lowry *et al.* (1951) using BSA (Fraction V)[5] as a standard.

Mesotocin Binding Assay

The labeling of MT with ^{125}I was performed by the Iodogen method (Takahashi *et al.*, 1992). Specific activity of [^{125}I]MT was 1,875 to 2,483 Ci/mmol determined by the method of Copeland *et al.* (1979). In the binding assay, polypropylene tubes used were pretreated overnight at 4 C with TE buffer containing 1% BSA. Aliquots of the membrane fraction (5 μg protein/200 μL per tube) were incubated at 30 C for 5 h with [^{125}I]MT (0.02 to 2.4 n*M*) in the presence (for nonspecific bindings) or absence (for total bindings) of 1 μ*M* of unlabeled MT in a total volume of 300 μL. To examine the binding specificity, unlabeled MT,[6] oxytocin (OT),[6] AVT,[6] arginine vasopressin (AVP),[6] chicken luteinizing hormone-releasing hormone-I (cLHRH-I: Gln^8-GnRH),[7] chicken LHRH-II (His,^{5}Typ,^{7}Tyr^8-GnRH),[6] and chicken angiotensin-II (cAngiotensin-II: Val^5-angiotensin-II),[6] were used as competitors. Concentrations of unlabeled peptides used were 0.006 to 6 μ*M* in MT, OT, AVT, and AVP, and 0.06 to 6 μ*M* in cLHRH-I, cLHRH-II, and cAngiotensin-II. Bound and free ligands were separated by centrifugation (10,000 × *g*, 20 min, 4 C). The radioactivity of the precipitate (bound ligand) was measured by a gamma counter (Packard Cobra).[8] The counting efficiency was 69 to 84%. Specific bindings were obtained by subtracting the nonspecific binding from the total binding and expressed as moles per milligram of protein. The equilibrium dissociation constant (K_d) and the maximum binding capacity (B_{max}) were determined by the method of Scatchard(H (1949).

Statistical Analyses

A half-maximal inhibition (ID_{50}) of [^{125}I]MT bindings was estimated by the use of a log-logit linear regression (Finney, 1964). The data were analyzed by one-way ANOVA (Snedecor and Cochran, 1967). When significant ($P < 0.05$) effects were found, Tukey's multiple range test (Tukey, 1953) was used to compare means of more than two groups.

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[8]Packard Instrument Co., Meriden, CT 06450.

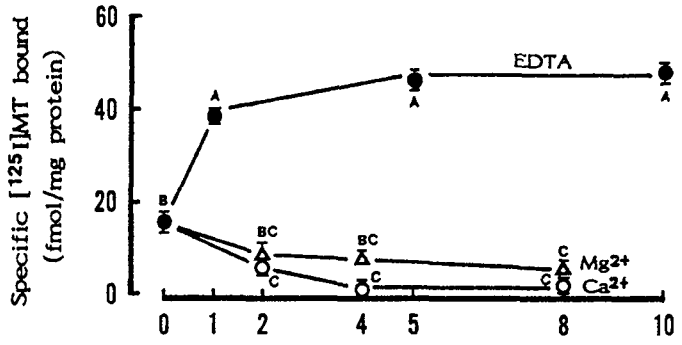

FIGURE 1. Relationship of specific bindings for [^{125}I]mesotocin (MT) to the concentration of EDTA, Mg^{2+}, or Ca^{2+} in the plasma membrane fractions of the kidney of hens. Samples (5 μg protein per tube) were incubated in 50 m*M* Tris buffer (pH 7.4) containing various concentrations of EDTA (•), Mg^{2+} (△), or Ca^{2+} (○) at 30 C for 5 h with 0.6 n*M* [^{125}I]MT in the absence or presence of 1 μ*M* unlabeled MT, and specific [^{125}I]MT bindings were measured. Each point represents the mean ± SEM of three separate pools of samples. Points that have different letters are significantly different ($P < 0.01$) by Tukey's test.

RESULTS

Effects of Cations, Incubation Temperature and Period, and Membrane Protein Concentration on Mesotocin Binding

The specific [^{125}I]MT binding was decreased by the presence of Mg^{2+} (8 m*M*) and Ca^{2+} (2 to 8 m*M*), and increased by the presence of EDTA (1 to 10 m*M*) (Figure 1). The binding at 30 C increased during the first 4 h of incubation, and then reached a plateau up to 8 h (Figure 2), but a remarkable increase was not observed at 4 C (Figure 2). A linear increase in the specific binding with the increase in the protein concentration from 2.5 to 20 μg per tube was observed when incubated at 30 C for 5 h (Figure 3).

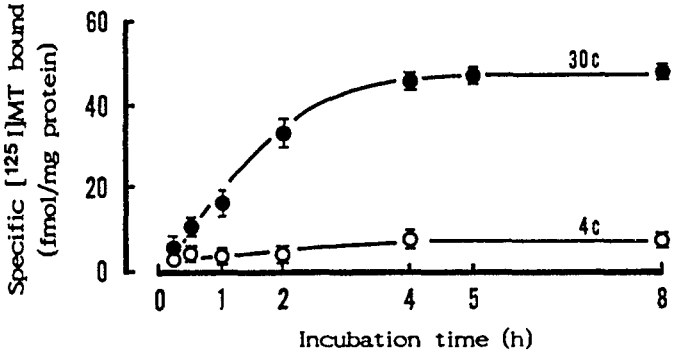

FIGURE 2. Time course of specific bindings for [^{125}I]mesotocin (MT) in the plasma membrane fractions of the kidney in hens. Samples (5 μg protein per tube) were incubated at 4 C (○) or 30 C (•) for various hours with 0.6 n*M* [^{125}I]MT in the absence or presence of 1 μ*M* unlabeled MT, and specific [^{125}I]MT bindings were measured. Each point represents the mean ± SEM of three separate pools of samples.

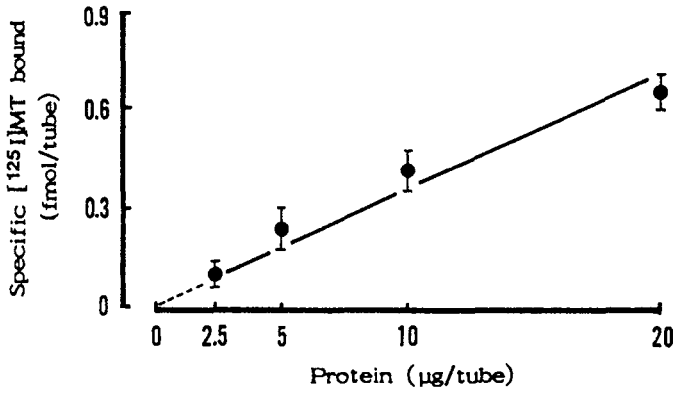

FIGURE 3. Relationship of specific bindings for [125I]mesotocin (MT) to the protein concentration in the plasma membrane fractions of the kidney of hens. Samples (2.5 to 20 μg protein per tube) were incubated at 30 C for 5 h with 0.6 n*M* [125I]MT in the absence or presence of 1 μ*M* unlabeled MT, and specific [125I]MT bindings were measured. Each point represents the mean ± SEM of three separate pools of samples.

Binding Specificity

The [125I]MT binding was markedly reduced by the presence of a 1,000-fold molar excess of unlabeled MT (0.6 μ*M*) and the presence of a 10,000-fold molar excess (6 μ*M*) of unlabeled OT and AVP (Figure 4), but was not affected by the presence of an equivalent molar excess of unlabeled cLHRH-I, cLHRH-II, and cAngiotensin-II (Figure 4). The AVT reduced the binding to about 60% when a 10,000-fold molar excess (6 μ*M*) was added (Figure 4). The half maximal inhibition (ID_{50}) value calculated from the data of dose-inhibition curve was 0.07 μ*M* for MT, 8.3 μ*M* for AVT, 0.38 μ*M* for OT, and 1.5 μ*M* for AVP.

Binding Affinity and Capacity

The specific [125I]MT binding increased with the increase in the amount of [125I]MT and reached a plateau at about 0.15 n*M*, but again increased and reached another plateau at about 1.2 n*M* (Figure 5). Scatchard analysis of the data revealed a curvilinear relationship between the amount of specific [125I]MT bindings and the ratio (B:F) of specific binding to free [125I]MT (Figure 5). The plot was fitted to two straight lines, suggesting the presence of two classes of binding sites: high and low affinity sites. The value of K_d and B_{max} obtained from five separate pools of samples were 0.08 ± 0.01 ($\bar{x}$ ± SEM) n*M* and 42 ± 4 fmol/mg protein for high affinity sites, and 0.87 ± 0.08 n*M* and 129 ± 6 fmol/mg protein for low affinity sites, respectively.

DISCUSSION

The membrane fractions of the kidney of the hen were found to contain a MT binding component (Figures 1 to 3). The specific [125I]MT binding was decreased by the presence of Ca^{2+} and Mg^{2+}, and increased by the presence of EDTA (Figure 1). These findings were similar to those on the AVT receptor of the uterus (shell gland) of the hen (Takahashi *et al.*, 1992), but different from those on the OT receptor of the mammalian uterus. The OT receptor binding in the mammalian uterus was increased by divalent cations (Ayad and Wathes, 1989) and decreased by EDTA (Sheldrick and Flint, 1985).

The binding to [125I]MT obtained from the competitive binding studies (Figure 4) was shown to be reduced by the presence of the unlabeled avian neurohypophysial hormones, MT and AVT, and also by the presence of unlabeled mammalian neurohypophysial hormones, OT and AVP, but not to be reduced by the presence of the other neuropeptides. Based on the ID_{50} values obtained from the data of Figure 4, the relative potency of the neurohypophysial hormones was in the following descending order: MT, OT, AVP, and AVT. Mesotocin was 118 times more potent than AVT, 21 times more potent than AVP, and 5 times more potent than OT. The results suggest that the discrepancy of the ID_{50} values between MT and AVT in the avian neurohypophysial hormones may be due to a specificity for the MT binding component in the kidney of the chicken.

The Scatchard analysis of saturation studies (Figure 5) revealed that the MT binding component possessed a curvilinear profile, indicating the presence of two distinct binding sites of low and high affinity. The equilibrium dissociation constant (K_d: indicating the degree of binding affinity) was 0.08 ± 0.01 n*M* in the high affinity site and 0.87 ± 0.08 n*M* in the low affinity site. The presence of two binding sites for MT is different from the finding reported on the mammalian renal AVP receptor possessing a single affinity binding site with the K_d value of 0.5 to 4.2 n*M* (Crause and

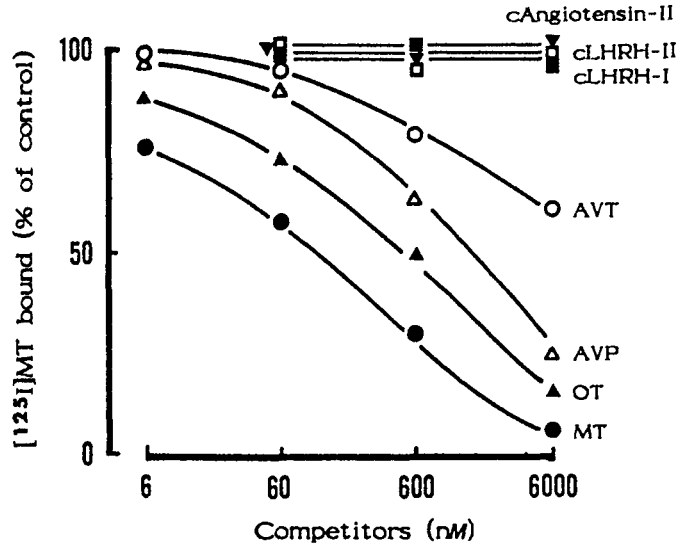

FIGURE 4. Competition for [125I]mesotocin (MT) bindings in plasma membrane fractions of the kidney in hens. Samples (5 μg protein per tube) were incubated at 30 C for 5 h with 0.6 n*M* [125I]MT in the absence (control) or presence of various fold molar excess of unlabeled MT (●), arginine vasotocin (AVT; ○), oxytocin (OT; ▲), arginine vasopressin (AVP; △), chicken luteinizing hormone-releasing hormone-I (cLHRH-I; ■), cLHRH-II (□), or chicken angiotensin-II (cAngiotensin-II; ▼). In control value, amounts of [125I]MT binding were 52 fmol/mg protein. Each point represents the mean of two separate pools of samples.

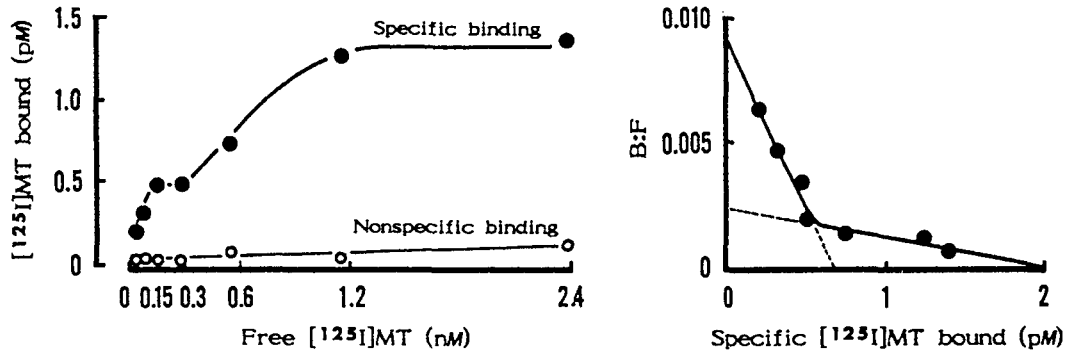

FIGURE 5. Saturation curve and Scatchard plot of specific bindings for [^{125}I]mesotocin (MT) in plasma membrane fractions of the kidney in hens. Samples (5 μg protein per tube) were incubated at 30 C for 5 h with various concentrations of [^{125}I]MT in the absence or presence of 1 μM unlabeled MT, and specific [^{125}I]MT bindings were measured. Values of equilibrium dissociation constant (K_d) were 0.08 and 0.90 nM and maximum binding capacity (B_{max}) values were 41 and 121 fmol/mg protein as determined by Scatchard analysis (1949). Each point represents the mean of duplicate determinations from one pooled sample. (•) Specific binding, (○) Nonspecific binding. B = bound; F = free.

Fahrenholz, 1982; Assimacopoulos-Jeannet *et al.*, 1983; Dorsa *et al.*, 1983; Cornett and Dorsa, 1985; Phillips *et al.*, 1990). In the oviduct magnum, isthmus, uterus, and vagina of the hen, the binding site for MT is a single class (Takahashi *et al.*, 1993), and the K_d value is similar to that in the low affinity site in the kidney obtained in the present study. The results in the present study suggest the presence of two distinct MT receptors in the kidney of the chicken.

The values of the binding affinity (K_d) expressed as moles per liter obtained in the present study was of the order of 10^{-11} in the higher affinity site, and of 10^{-10} in the lower affinity site. The intact chicken blood MT concentration expressed as moles per liter is at the level of 10^{-11} (Nouwen *et al.*, 1984; Koike *et al.*, 1986; Robinzon *et al.*, 1988, 1990; Shimada *et al.*, 1991). The concentration is almost at the same level as the K_d value of the higher affinity binding site. This result means that MT is capable of binding to the higher affinity site of the MT receptor of the kidney, but not to the lower affinity site. The blood MT concentration in the chicken was increased by dehydration (Nouwen *et al.*, 1984) or infusion of hypoosmotic saline (Koike *et al.*, 1986). When a lower dose of MT (0.0025 to 0.25 μg/kg body weight) is injected into chickens, the volume of urine excreted is decreased as in the case of AVT. However, when a higher dose (2.5 to 25 μg/kg body weight) is injected, the urine volume increases unlike the case of AVT (Takahashi *et al.*, 1995). It seems likely that the presence of two distinct MT receptors found in the present study may be related to the two reciprocal effects of MT, one is antidiuretic and the other is diuretic, on the kidney of the hen.

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