Characterization of the Metal Ion Requirement for Oxytocin-Receptor Interaction in Rat Mammary Gland Membranes*

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The presence of a divalent cation is essential for the specific binding of [3H]oxytocin to particulate receptor preparations of the mammary gland of the lactating rat. Oxytocin binding was potentiated in increasing order by divalent zinc, magnesium, nickel, manganese, and cobalt, but was negligible in the presence of divalent calcium, copper, and iron. The apparent K∞ values for oxytocin-receptor interaction in the presence of optimal concentrations of metal ions ranged from 3.1 to 8.6 × 10^-7 M^-1. The metal ions did not affect site-site interactions among oxytocin receptors, as evidenced by linear Scatchard plots, Hill coefficients of 1, and the lack of effect of unbound oxytocin on the dissociation rate constant of the oxytocin-receptor complex. The kinetics of the association and dissociation of the hormone-receptor complex showed a fast step for the binding of metal ion followed by a slow rate-determining step for the binding of oxytocin.

The active divalent metal ions appear to affect oxytocin binding by two separate processes. 1) Increasing concentrations of divalent nickel, magnesium, and manganese caused an increase in the concentration of binding sites available for oxytocin, while the affinity for the hormone was not changed. 2) Increasing amounts of cobalt increased the affinity of the receptor site for oxytocin, but did not affect the concentration of sites available for oxytocin binding. Because combinations of maximal concentrations of both types of metal ions were not additive with respect to the concentration and affinity of oxytocin-binding sites, metal ions appear to bind to identical sites.

We postulate that the oxytocin receptor possesses two distinct regions for metal ion interaction. The binding of metal ion to Region A (availability) results in a fast step for the binding of oxytocin. The active divalent metal ions appear to affect oxytocin binding by two separate processes. The binding of metal ion to Region B (binding) results in a receptor of high affinity and full availability for oxytocin, while the affinity for oxytocin was not changed. The combined effects of metal ions on the binding of oxytocin were not additive with respect to the concentration and affinity of sites available for oxytocin binding. Therefore, the binding of metal ions to both sites resulted in a receptor of high affinity and full availability, consistent with the results of studies of neurohypophysial hormone action on isolated target tissues.

Divalent magnesium is important for the optimal contractile activities of the neurohypophysial hormones and their analogues on the myometrium (1-7), vascular smooth muscle (8, 9), and isolated mammary strips (10). On the other hand, Mg^2+ does not potentiate the vasoconstrictor actions of serotonin, angiotensin, or epinephrine (8). Thus, the actions of Mg^2+ on smooth muscle appear to be unique for neurohypophysial peptides.

The binding of [3H]oxytocin to mammary particulate fractions reflects oxytocin-receptor interaction (10-12). This binding requires the presence of a divalent cation other than Ca^2+; Soloff and Swartz (10) have shown that the order of effectiveness is Co^2+ > Mn^2+ > Mg^2+ > Zn^2+. Monovalent cations such as Na^+, Li^+, and K^+ do not appear to affect oxytocin-receptor interaction (10). Addition of EDTA resulted in dissociation of [3H]oxytocin from its binding sites; inhibition of oxytocin binding occurred in the presence of EDTA and could be reversed by removal of EDTA and addition of a divalent metal ion (10). Thus, the binding site is not irreversibly altered by the addition of metal ion or by its absence.

This study is the first detailed examination of the mechanism of potentiation of peptide hormone-receptor binding by divalent metal ions. A unique model has been developed to account for these effects in broken cell preparations as well as in tissues.

EXPERIMENTAL PROCEDURES

Materials—[tyrosyl-3H]Oxytocin, either purchased from Schwarz/Mann (31 Ci/mmol) or a gift (34 Ci/mmol) from Luis Branda, McMaster University, has been characterized (10, 13). The tritiated hormone was aliquoted into plastic tubes and stored at -80°C. Oxytocin was a gift from Sandoz, Ltd. All other chemicals were analytical grade.

Mammary particulate fractions were prepared from lactating rats (ARS/Sprague-Dawley, Madison, Wis.) 12 to 14 days postpartum, as described previously (10). The abdominal mammary glands were freed of fascia, minced, and homogenized in 10 volumes of Tyrode solution in a conical glass homogenizer at 4°C. The samples were then homogenized in a Polytron PT-10 (Brinkmann Instruments) for 10 s. The homogenate was centrifuged for 10 min at 1,000 × g, the supernatant was filtered through surgical gauze to remove clumps of fat, and the filtrate was centrifuged at 97,000 × g for 50 min. The resulting pellet was resuspended in a volume of Tyrode solution equal to one-half the original weight of the tissue, and aliquots of the suspension were stored at -80°C. Protein concentrations were determined by the method of Lowry et al. (14) with bovine serum albumin as the standard; the aliquots contained approximately 20 to 23 mg of protein/ml.

Determination of the Binding of [3H]Oxytocin to Mammary Particles in the Presence of Metal Ions—The frozen suspensions of rat mammary particles were thawed and centrifuged at 170,000 × g for 30 min, the supernatants were discarded, and the pellets were resuspended in metal-free buffer (50 mm Tris/maleate, pH 7.6, containing 0.1% gelatin and 1 mm EDTA). After 15 min at 4°C, the suspensions were centrifuged again for 30 min at 170,000 × g. The pellets were resuspended and centrifuged in metal-free buffer to remove any remaining traces of EDTA; this procedure was repeated. The rinsed pellets were resuspended in buffer (6 mg of protein/ml) containing the appropriate concentration of metal ion. Combinations of [3H]-
oxytocin and nonradioactive hormone were mixed with the particulate preparations and incubated at 20°C for 1 h. Nonspecific binding was determined by measuring the amount of [3H]oxytocin bound in the presence of 50 nM nonradioactive oxytocin. The reactions were terminated by the addition of 5 ml of ice cold buffer (metal-free and without gelatin) to each sample. The particulate material was isolated by filtration on a Millipore sampling manifold over a combination of prewetted filters, MF-Millipore 8 μm (SC) and 0.3 μ (PH). The time for filtration never exceeded 30 s. Each filter was then rinsed twice with 0.5 ml of ice cold metal-free, gelatin-free, Tris buffer. The dissociation of hormone was negligible under these rinsing conditions.

The amount of radioactivity bound to the isolated particulate material was determined by burning the filters in a Packard sample oxidizer and counting with a liquid scintillation spectrometer. Recovery of tritium as H2O after combustion was >97%. The efficiency of counting, approximately 30%, was determined by the channels ratio method.

The equilibrium binding studies were carried out with Tris buffer. Computations, utilizing the known association constants of Tris and the divergent metal ions used in this study, showed that approximately 16% of the Ni2+, 45% of the Co2+, 32% of the Zn2+, and 95% of the Mn2+ and Mg2+ exist as the free metal ion in a 50 mM Tris solution (15-17). The concentrations referred to in the text and figures are of total metal ion, unless otherwise stated.

**Association Rate Studies**—For the association rate studies, 5.2 ml of particulate material was allowed to equilibrate to 20°C in a water-jacketed vessel and at t = 0, the appropriate amount of hormone was added in 0.5 ml of buffer. Aliquots of 200 μl were removed from the reaction vessel at intervals and placed immediately into test tubes containing 5 ml of ice cold buffer. Each sample was then filtered and assayed for specifically bound oxytocin.

**Dissociation Rate Studies**—After equilibration of a particulate preparation and hormone for 30 min in a water-jacketed vessel at 20°C, the solution was diluted 1:50 with the appropriate buffer solution in the presence or absence of a 100 fold excess of nonradioactive oxytocin. Aliquots were removed at intervals and filtered immediately. Bound radioactivity was determined as described above.

**Binding of Metal Ions to Oxytocin**—Approximate binding constants of Zn2+, Co2+, Mg2+, Ca2+, and Ni2+ complexation with oxytocin were estimated by the ability of each metal ion to displace Cu2+ from oxytocin. The free Cu2+ concentration was determined with a solid state Cu2+ electrode (Orion). The electrode was calibrated with a stock solution of Cu(NO3)2 and corrected for the presence of CuOH.

First, the decrease in free Cu2+ concentration in the presence of oxytocin was measured at 25°C, pH 6.5. Next, the appropriate metal ion was added until an increase in free Cu2+ concentration was noted. These measurements are based on the assumption that the binding sites on the oxytocin molecule for Cu2+ are similar to those for the other divalent metal ions and the values obtained are considered approximations of the true binding constants.

**RESULTS**

**Specific Binding of Oxytocin with Different Metal Ions**—As shown in previous studies with particulate fractions (10) and isolated cells (18) from the mammary gland of the lactating rat, the interaction of oxytocin and receptor sites results in linear Scatchard plots. The binding of oxytocin to mammary receptor sites in the presence of increasing concentrations of metal ions has been examined with Co2+, Mn2+, Ni2+, Mg2+, Zn2+, Ca2+, and Cu2+ and analyzed according to Scatchard (19). Oxytocin binding was indistinguishable from nonspecific binding in the presence of Mn2+ (0 to 10 mM), Ca2+ (0 to 5 mM), and Cu2+ (0 to 5 mM), but was potentiated by Co2+, Mn2+, Ni2+, and Mg2+ (Fig. 1). The binding affinity for oxytocin was maximal with 5 mM Co2+, apparent $K_d = 8.6 \times 10^8$ M$^{-1}$.

The apparent $K_d$ values with 5 mM Mn2+, Mg2+, and Ni2+ were 4.7, 4.3, and 3.1 x 10$^8$ M$^{-1}$, respectively. The amount of oxytocin bound to 6 mg of particulate protein/ml was near maximal with concentrations of Mn2+ near 5 mM; the value of $R_{max}$ with 5 mM Mn2+ was 0.85 nm compared with the theoretical maximum of 1.05 nm shown in the inset to Fig. 1. A decrease in the concentration of either Mn2+, Mg2+, or Ni2+ resulted in a reduction in the maximum number of available binding sites for oxytocin; the apparent $K_d$ values, as indicated by the slopes of the Scatchard plots, were not different within experimental error (Fig. 1). In contrast, with 0.5 to 5 mM Co2+, the maximum number of binding sites available for oxytocin remained constant, but the affinity of the receptor sites for the hormone decreased as the [Co2+] was reduced (Fig. 1).

Scatchard plots obtained with [3H]oxytocin alone were identical with those determined with combinations of labeled and unlabeled oxytocin. Therefore, radiolabeled oxytocin was indistinguishable from unlabeled hormone.

To determine whether metal ions affect site-site interactions among the oxytocin-binding sites, the equilibrium binding data were plotted according to the Hill transformation (20). Plots on a logarithmic scale of $B/(B_{max} - B)$ versus unbound oxytocin, where $B$ and $B_{max}$ are the concentrations of oxytocin...
bound with a given and saturating concentration of hormone, respectively, gave Hill coefficients that were close to 1 (0.89 to 1.07) with correlation coefficients greater than 0.96 (Fig. 2). Parallel lines were obtained with different concentrations of Co²⁺, whereas a single line was obtained with increasing concentrations of Mn²⁺ and Mg²⁺ (Fig. 2). The linear Scatchard plots and Hill coefficients of 1 indicate that oxytocin is bound to a single class of binding sites that show no significant cooperativity in the presence of metal ions.

**Dissociation Rate Studies with Mn²⁺**—The dissociation of the oxytocin-mammary receptor complex was studied by diluting an equilibrium mixture of the bound hormone either with buffer alone or with buffer containing nonradioactive hormone. Under these conditions, reassociation of the hormone-receptor complex was negligible and the data could be analyzed by assuming a unimolecular dissociation where

\[
R + \text{Oxy} \rightleftharpoons R\cdot\text{Oxy}
\]  

For this mechanism

\[
k_t = \ln \frac{1}{2} / t_{1/2}
\]

where \( t_{1/2} \) is the half-time for the dissociation determined by plotting the log of the percentage of dissociation versus time. The half-time for a series of dissociation experiments was 15.2 ± 3.1 min with initial oxytocin concentration between 5 and 50 nM (Table I). In one experiment, the initial receptor concentration was halved with no significant change in \( t_{1/2} \) (Table I). No significant differences in dissociation rates were found with or without a 100-fold excess of nonradioactive oxytocin in the diluent buffer (Table I).

Because the dissociation rates were not accelerated by the presence of an excess of oxytocin, retention of free-hormone in an insoluble compartment is not a rate-determining feature of this system (21). Thus, we can eliminate any effect on the kinetics due to ligand distribution between the soluble and insoluble phases.

**Association Rate Studies with Mn²⁺**—The specific binding of oxytocin to the mammary particulate fraction at 20°C with 5 mM Mn²⁺ was time-dependent and was proportional to both the concentration of receptor (Fig. 3A) and of oxytocin (Fig. 3B). This suggests that the binding of oxytocin to the particulate receptor is a second order process. The mechanism which fits the data is shown below:

\[
R + \text{Oxy} \rightleftharpoons R\cdot\text{Oxy}
\]

where \( R \) is the receptor site, \( \text{Oxy} \) is oxytocin, and \( R\cdot\text{Oxy} \) is the complex formed. Determinations of \( k_t \) were made at early times (0 to 5 min) to minimize contributions from the dissociation reaction. The integrated equation for this mechanism, including a term for the dissociation reaction, may be expressed as

\[
\frac{1}{q} \ln \frac{[R\cdot\text{Oxy}]}{[R] + [\text{Oxy}]} = \frac{1}{2} \left( \frac{\beta - q^{1/2}}{\beta + q^{1/2}} \right) k_t t
\]

where \( q = \beta^2 - 4\alpha \), \( \beta = -(k_r + R^0 + \text{Oxy}^0) \), and \( \alpha = (R^0) \).

| [Oxytocin]* | N | \( t_{1/2} \) (±S.E.) |
|------------|---|-------------------|
| 5 nM       | 8 | 17.1 (±2.5)       |
| 5 b        | 2 | 14.4, 19.4        |
| 10 nM      | 2 | 11.3, 12.7        |
| 10 b       | 1 | 11.5              |
| 20 nM      | 2 | 10.0, 15.6        |
| 20 b       | 1 | 10.0              |
| 25 nM      | 1 | 16.0              |
| 50 nM      | 1 | 13.7              |
| 10 (½ R)*  | 1 | 16.1              |
| Total      | 19| 15.2 (±3.1)       |

*Initial hormone concentration.

**Table I**

Dissociation half-time for oxytocin binding to mammary receptor site with 5 mM Mn²⁺

\* One-half receptor concentration.

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**Fig. 2.** Hill plots of the specific binding of oxytocin to the mammary receptor site with increasing Co²⁺ ( ), 5 mM; ( ), 1 mM; ( ), 0.5 mM; ( ), 0.1 mM); Mn²⁺ ( ), 5 mM; ( ), 1 mM; ( ), 0.5 mM; and Mg²⁺ ( ), 5 mM; ( ), 1 mM). For Co²⁺, the slopes of the lines for 5, 1, 0.5, and 0.1 mM were 0.93 (correlation coefficient, 0.93), 0.99 (correlation coefficient, 0.998), 0.98 (correlation coefficient, 0.982), 0.89 (correlation coefficient, 0.998), respectively. For Mn²⁺ and Mg²⁺, the slopes were 0.58 (correlation coefficient, 0.956) and 1.07 (correlation coefficient, 0.996), respectively.
Metal Ions and Oxytocin-Mammary Receptor Interaction

(Oxy)° and where the superscript zero indicates initial concentrations and the subscript t indicates the concentration at the time of the measurement. The relationship between the left-hand term of the integrated rate equation, $F(c)$, and $t$ was linear during the first 5 min of association. Association rates also were linear during the first 5 min with the concentrations of Co⁺⁺, Ni⁺⁺, and Mg²⁺ shown in Fig. 1. Analysis of the data by assuming a two-step mechanism by the method of Strickland et al. (22) gave rate constants that were internally inconsistent. The average forward rate constant with 5 mM Mn²⁺ for five experiments in which the initial oxytocin concentration was varied was $3.7 \pm 0.3$ (S.E.) $\times 10^5$ M⁻¹ s⁻¹ (Table II).

### Kinetic Data with Other Metal Ions
We have examined the kinetics of association and dissociation of oxytocin in the presence of increasing amounts of Co⁺⁺, Mg⁺⁺, and Ni⁺⁺ as well as Mn⁺⁺ (Table III). The rates of association and dissociation of oxytocin were unchanged by increasing concentrations of Mn⁺⁺, Mg⁺⁺, and Ni⁺⁺ (Table III). These findings are consistent with the results from studies of the binding under steady state conditions; namely, these metal ions caused an increase in the concentration of receptor sites but did not affect the magnitude of the equilibrium association constant. In contrast, increasing concentrations of Co⁺⁺ caused a small increase in the association rate constant and a decrease in the dissociation rate constant. These effects of increasing cobalt concentration on the rate constants may account, in part, for the increase in stability of the oxytocin receptor complex in the presence of increasing amounts of cobalt.

**Delayed Additions of Metal Ion**—The binding of [³²H]oxytocin to a mammary receptor preparation was negligible for up to 30 min in the absence of metal ion (Fig. 4). The addition of Mn⁺⁺, to a final concentration of 5 mM, resulted in rapid interaction of oxytocin and receptor with an association rate constant identical with that obtained when [³²H]oxytocin and Mn⁺⁺ were added simultaneously (Fig. 4).

**Effect of Metal Ion on the Stability of the Oxytocin-Receptor Complex**. The importance of the metal ion in stabilizing the oxytocin-receptor complex was studied by comparing the dissociation rates of oxytocin in the presence or absence of free metal ion. Dissociation of a pre-equilibrated mixture of oxytocin, receptor and 5 mM Co⁺⁺ or Mn⁺⁺ was initiated by a 1:50 dilution with buffer containing either 5 mM metal ion, no metal ion, or no metal ion and 1 mM EDTA.

### Table II

| [Oxytocin]° | $k_{a}$ (±S.E.) | $k_{d}$ (±S.E.) |
|------------|----------------|----------------|
| nM         | $M^{-1}$ s⁻¹  | $M^{-1}$ s⁻¹  |
| 1.1        | 3.2 (±0.3) $\times 10^5$ | 0.8 (±0.8) |
| 3.6        | 3.8 (±0.4) $\times 10^5$ | 0.7 (±0.4) |
| 7.8        | 3.5 (±0.3) $\times 10^5$ | 0.8 (±0.4) |
| 17.1       | 3.9 (±0.4) $\times 10^5$ | 1.1 (±0.4) |
| 30.0       | 4.0 (±0.4) $\times 10^5$ | 1.2 (±0.4) |
| Total      | 3.7 (±0.3) $\times 10^5$ | 1.0 (±0.3) |

*Initial concentration.

*Calculated using the equation shown in the text. The standard error represents the standard error for the slope of the best fit straight line.

### Table III

| Metal  | Total concentration | Actual free concentration | $K_{a} \times 10^{5}$ ± S.E. | Concentration of sites | $k_{a} \times 10^{5}$ ± S.E. | $k_{d} \times 10^{5}$ ± S.E. |
|--------|---------------------|--------------------------|-------------------------------|------------------------|-----------------------------|-----------------------------|
|        | mM                  | mM                       | $M^{-1}$                      | nM                    | $M^{-1}$ s⁻¹               | $M^{-1}$ s⁻¹               |
| Co⁺⁺   | 0.5                 | 0.22                     | 2.9 ± 0.8                     | 0.89                   | 2.1 ± 0.1                  | 2.0                         |
|        | 1.0                 | 0.45                     | 5.4 ± 0.3                     | 0.89                   | 2.9 ± 0.2                  | 1.8                         |
|        | 5.0                 | 2.30                     | 8.6 ± 0.9                     | 0.84                   | 2.7 ± 0.7                  | 1.2                         |
| Mn⁺⁺   | 0.5                 | 0.48                     | 2.1 ± 1.0                     | 0.38                   | 3.9 ± 0.2                  | 0.8                         |
|        | 1.0                 | 0.95                     | 2.4 ± 0.7                     | 0.66                   | 3.9 ± 0.2                  | 0.9                         |
|        | 5.0                 | 4.80                     | 4.7 ± 0.6                     | 0.84                   | 3.7 ± 0.3                  | 0.8                         |
| Mg⁺⁺   | 1.0                 | ab                      | 4.1 ± 0.1                     | 0.44                   | 4.7 ± 0.3                  | 2.4                         |
|        | 5.0                 | ab                      | 4.3 ± 0.1                     | 0.59                   | 3.8 ± 0.1                  | 1.9                         |
| Ni⁺⁺   | 5.0                 | 0.84                     | 3.1 ± 0.3                     | 0.74                   | 4.4 ± 0.1                  | 2.0                         |
|        | 10.0                | 1.90                     | 2.8 ± 0.7                     | 0.97                   | 6.1 ± 0.4                  | 1.6                         |

*The amount of metal ion complexed to Tris was calculated using the association constants given in Refs. 15, 16, and 17.

* Mg⁺⁺ binds to Tris with an affinity lower than that of Mn⁺⁺.

![Fig. 3. Time course of specific oxytocin binding at 20°C to a rat mammary particulate preparation as a function of A, receptor concentration (O, 6 mg of protein/ml; ●, 3 mg of protein/ml; oxytocin, 9.8 nM); B, initial oxytocin concentration (initial oxytocin O, 9.8 nM; □, 4.8 nM, △, 1.3 nM; 6 mg of protein/ml).](http://www.jbc.org/ by guest on March 18, 2020)
buffer containing 5 mM of the appropriate metal ion resulted in no experimentally detectable dissociation of oxytocin by 1 min; however, dilution with buffer containing no metal ion or containing no metal ion and 1 mM EDTA resulted in the dissociation by 1 min of 43% and 72% to 79%, respectively, of the oxytocin bound (Fig. 5).

Metal Ion Binding to Oxytocin—To assess the affinity of oxytocin for metal ions, we determined the overall binding constants of a series of divalent metal ions. The results of pH titration studies indicated that the affinity of most of the divalent metal ions for oxytocin was too low to measure with reasonable amounts of hormone by this technique. Copper binds to both oxytocin and vasopressin, with a binding constant of about $5 \times 10^5$ M$^{-1}$ at pH 7 (23). We used a solid state Cu$^{2+}$ electrode to measure the binding of Cu$^{2+}$ to oxytocin. We then added another divalent metal ion and determined the relative binding constant for the metal ion based on its ability to displace Cu$^{2+}$ from oxytocin (Table IV). Although these values are only approximate, they indicate clearly the relatively low affinity of oxytocin for most divalent metal ions.

Table IV

| Metal Ion | $K_c$ (M$^{-1}$) |
|-----------|-----------------|
| Cu$^{2+}$ | $9 \times 10^4$ |
| Zn$^{2+}$ | $5 \times 10^3$ |
| Co$^{2+}$ | $4 \times 10^2$ |
| Mg$^{2+}$ | $3 \times 10^3$ |
| Ca$^{2+}$ | $2 \times 10^3$ |
| Ni$^{2+}$ | $2 \times 10^3$ |

Association of Oxytocin to Mammary Membrane Particles with Combinations of Divalent Metal Ions—To determine whether the different metal ions bind to the same site when enhancing oxytocin binding, we examined combinations of maximal concentrations of metal ions with 5 mM Mn$^{2+}$ and 5 mM Co$^{2+}$. The results are plotted as 1/bound oxytocin (1/B) versus 1/free oxytocin (1/F) for either Co$^{2+}$ or Mn$^{2+}$ with Mn$^{2+}$ or Co$^{2+}$, Ni$^{2+}$, and Mg$^{2+}$ as the second metal ion (Fig. 6). Specific binding was not significantly increased by maximal combinations of metal ions when compared with either Mn$^{2+}$ or Co$^{2+}$ alone (Fig. 6).

Effect of Ca$^{2+}$ on the Binding of Oxytocin in the Presence of 5 mM Mg$^{2+}$—Calcium is required for the contractile response of mammary strips to oxytocin (8, 24, 25). Because the

![Fig. 4](http://example.com/fig4.png)

**Fig. 4.** The effect of 5 mM Mn$^{2+}$ on the time course of specific association of oxytocin with the mammary receptor. The metal ion was added either simultaneously with the hormone (A) or after a delay of 30 min (B). The initial oxytocin concentration was 3.5 nM; 6 mg of particulate protein/ml.

![Fig. 5](http://example.com/fig5.png)

**Fig. 5.** Dissociation of oxytocin from an equilibrated mixture of 5 mM metal ion, oxytocin (4 nM), and mammary particulate preparation. The dissociation was initiated by 1:50 dilution in buffer containing 5 mM metal ion (A), buffer without metal ion (B), and buffer with 1 mM EDTA and no metal ion (C).

![Fig. 6](http://example.com/fig6.png)

**Fig. 6.** A double reciprocal plot of oxytocin specifically bound (B) versus free oxytocin (F) with a mammary receptor preparation (6 mg of protein/ml). Upper panel: 5 mM Co$^{2+}$, empty circle; 5 mM Co$^{2+}$ and 5 mM Mn$^{2+}$, empty triangle; and 5 mM Co$^{2+}$ and 5 mM Mg$^{2+}$, empty square. Lower panel: 5 mM Mn$^{2+}$, solid circle; 5 mM Mn$^{2+}$ and 5 mM Co$^{2+}$, solid triangle; and 5 mM Mn$^{2+}$ and 5 mM Ni$^{2+}$, solid square.
binding of oxytocin to mammary receptor sites was not affected by Ca" (Fig. 7, inset). Ca" appears to be involved in molecular events distal to the activation of the receptor. However, the binding of [3H]oxytocin to mammary receptor sites with 5 mM Mg" was inhibited by 0.2 to 1.0 mM Ca" (Fig. 7). Concentrations of Ca" greater than 1 mM were not inhibitory. Scatchard plots of oxytocin binding with 5 mM Mg" and increasing concentrations of Ca" indicate that the inhibitory activity of 0.2 to 0.6 mM Ca" was due to a reduction in the concentration of binding sites available for oxytocin (Fig. 8). The affinity of the receptor sites for oxytocin was not changed, as shown by the parallel Scatchard plots for 0, 0.2 and 0.4, and 0.6 mM Ca". In agreement with these findings, association and dissociation rates of the hormone-receptor complex in the presence of 5 mM Mg" were not affected by Ca" concentrations of 0.5 and 0.6 mM.

Ca" (1 mM) caused an increase in the affinity of the receptor sites for oxytocin as well as a further decrease in the maximal concentration of receptor sites (Fig. 8). This increased affinity for oxytocin may explain why concentrations of Ca" greater than 1 mM were not inhibitory with the concentrations of oxytocin and receptor used (Fig. 7).

All of the Scatchard plots were linear (Fig. 8), which indicates that oxytocin was bound to a single class of independent sites at the concentrations of Ca" tested. The observation that calcium can affect both the concentration as well as the affinity of binding sites for oxytocin is consistent with the effects of other metal ions that we have examined.

**DISCUSSION**

In the absence of active divalent metal ions, specific oxytocin binding to mammary particulate material was only slightly greater than nonspecific binding levels. For example, in the absence of metal ion, the bound/free ratio of oxytocin never exceeded 0.05 when 0.01 to 0.1 nM oxytocin was bound (compare with data in Fig. 1). Thus, the presence of an appropriate divalent metal ion is an absolute requirement for significant oxytocin binding.

The effect of the metal ion was rapid compared with oxytocin binding. Within 10 s of the addition of Mn" to a pre-equilibrated mixture of receptor and oxytocin, hormone binding occurred at a rate identical with that obtained when oxytocin, metal ion, and receptor were incubated simultaneously (Fig. 4). Divalent metal ion complexation is known to occur rapidly with typical association rate constants of 2 x 10^6 to 3 x 10^7 M^-1 s^-1 (26). Metal ion was required to prevent dissociation of the hormone-receptor complex because dissociation of the oxytocin-receptor complex was greatly accelerated when metal ion was complexed with EDTA (Fig. 5).

In spite of the distinct differences between the effects of Co" and the other divalent cations, all of the divalent metal ions probably bind to the same sites because combinations of maximal amounts of Co" and other metal ions were not additive with respect to the concentration and affinity of oxytocin-binding sites (Fig. 6). Calcium alone did not affect oxytocin binding, but, in combination with 5 mM Mg", 0.2 to 0.6 mM Ca" caused a reduction in the concentration of oxytocin receptor sites (Fig. 8). Higher concentrations of Ca" in the presence of 5 mM Mg" increased the affinity of the receptor for oxytocin (Fig. 8). These findings indicate that, under certain conditions, a single metal ion, Ca", can affect both the concentration and the affinity of receptor sites for oxytocin. Therefore, any model describing the effects of metal ion must be consistent with this dual action of Ca".

Although metal ions may potentiate biological effects by modifying the conformation of the hormone directly, this is unlikely to be the case for the oxytocin-mammary receptor system. Neurohypophysial hormones form strong complexes with Cu" (23), yet copper is inactive in the oxytocin-mammary receptor system. Although those divalent metal ions that potentiate oxytocin-receptor interaction have a lower affinity than Cu" for oxytocin (Table IV), we estimate that, under most experimental conditions, about 95% of the oxytocin would be complexed with metal ion. It is unlikely, however, that the initial effect of the metal ion is upon the conformation of oxytocin. If metal ion binding to oxytocin were of significance, then

\[
M + Oxy = M \cdot Oxy + R \frac{k_{2a}}{k_{1a}} M \cdot Oxy \cdot R
\]  

(4)
where $M$ is the metal ion, $Oxy$ is oxytocin, $R$ is the receptor site, $M\cdot Oxy$ is the metal ion-oxytocin complex, and $M\cdot Oxy\cdot R$ is the complex of all three components; the equal sign indicates a fast pre-equilibrium (1 to 2) and the arrows indicate the rate-determining step (2 to 3). If the assumption is made that $M\cdot Oxy$ is present as a steady state intermediate and that the concentration of free receptor is in the nanomolar range, the observed forward and reverse rate constants may be expressed as

$$k_f = K_{12}K_{23} [M] \text{ and } k_r = k_{21}$$

where $K_{12}$ is the association constant for oxytocin-metal ion binding. Were Mechanism 4 correct, then the observed forward rate constant should be directly dependent on the concentration of free metal ion. However, there was no increase in the observed association rate constant with increasing Mn$^{2+}$ and Mg$^{2+}$ concentrations (Table III). With Co$^{2+}$, there was only a 26% increase in the observed forward rate constant with a 5-fold increase (1.0 to 5.0 mM) in cobalt concentration (Table III). Furthermore, Scatchard analysis of oxytocin binding in the presence of cobalt showed the same concentration of receptor available to bind oxytocin at all cobalt concentrations. The fact that cobalt concentration was not limiting in this regard eliminates Mechanism 4 as a realistic model. We conclude that metal ions bind to the receptor site first and then a complex is formed between the receptor site and the hormone. Our data cannot discriminate between a mechanism involving metal ion interaction with the receptor site only or metal ion interaction with both the receptor site and oxytocin. The Scatchard plots as well as the kinetic data are strong indications that receptor-metal ion interactions must occur prior to hormone binding.

We have developed a model that is consistent with our data in which the active metal ions affect the conformation of the receptor site for oxytocin (Fig. 9). The receptor complex is postulated to consist of at least two subunits or domains and to contain at least two distinct metal ion binding regions: $A$, for availability and $B$, for binding (Fig. 9, I). The $A$ site is part of an "inhibitory" subunit that blocks the access of oxytocin to the receptor binding site. When metal ions bind to the inhibitory subunit at Site $A$, the inhibitory subunit dissociates from the binding subunit and exposes the binding site for oxytocin (Fig. 9, II). Binding of metal ion to the second binding region, $B$, results in a conformational change at the hormone binding site (Fig. 9, III) and allows for high affinity oxytocin binding when the inhibitory subunit is dissociated (Fig. 9, IV). For the sake of simplicity, we have postulated that the inhibitory portion of the receptor complex is a distinct subunit. It is possible that both the $A$ and $B$ sites are on the same molecule.

The active metal ions can bind to both $A$ and $B$ regions. However, the $B$ region has a greater affinity for Mn$^{2+}$ and Mg$^{2+}$ than does the $A$ region, so that at low concentrations of these ions the $B$ region is occupied before the $A$ region. This results in a receptor site with a maximal affinity for oxytocin, but with limited accessibility because of the steric hindrance posed by the presence of an undissociated inhibitory subunit (Fig. 9, I $\rightarrow$ III). As the concentration of Mn$^{2+}$ or Mg$^{2+}$ increases, the $A$ sites on the receptor complex progressively become occupied. This results in a greater number of dissociated inhibitory subunits and, consequently, an increase in the availability of the high affinity sites for oxytocin (Fig. 9, III $\rightarrow$ IV). The increase in the total number of binding sites for oxytocin as the concentration of Mn$^{2+}$ or Mg$^{2+}$ is increased is proportional to the occupancy of the $A$ region by metal ion. This is reflected in the parallel shift to the right of the Scatchard plots for oxytocin-receptor binding in the presence of increasing amounts Mn$^{2+}$, Mg$^{2+}$, or Ni$^{2+}$ (Fig. 1).

Cobalt, on the other hand, has a greater affinity for the $A$ site, so that at low concentrations of Co$^{2+}$, the inhibitory subunit is dissociated; this results in the maximum availability of binding sites, but the receptor site is in a conformation that has a relatively low affinity for oxytocin (Fig. 9, I $\rightarrow$ II). As the concentration of Co$^{2+}$ is raised, the $B$ site progressively becomes occupied. This results in an increasing concentration of receptors with maximal affinity for oxytocin (Fig. 9, II $\rightarrow$ IV). The increase in affinity is proportional to the relative amount of receptor subunits binding metal ion at Site $B$. Thus, Scatchard plots of oxytocin binding to receptor with
increasing concentrations of Co²⁺ will show an increasing association constant for oxytocin binding with a maximal concentration of receptor sites always available for binding. Further support for our model is provided by the observation that Ca²⁺, while not effective by itself in potentiating the binding of oxytocin to mammary receptor sites, was able to exert separate effects on both the affinity and concentration of receptor sites. Calcium, 0.2 to 0.6 mM, reduced the concentration of accessible binding sites, presumably by competing with Mg²⁺ at the A metal binding region. Concentrations of Ca²⁺ greater than 1 mM caused an increased affinity of the receptor site for oxytocin. This effect may be either due to the direct interaction of Ca²⁺ with the B site or to the effect of Ca²⁺ on the concentration of Mg²⁺ available for binding to the B site.

The effects of combinations of metal ions near maximal concentrations were not additive (Fig. 6). These findings add further support to the model, which proposes that the divalent metal ions bind to identical regions, A and B, when enhancing oxytocin binding. Our model is consistent with the results of studies of neurophysiological hormone action on isolated target tissues. These studies have suggested that Mg²⁺ potentiates the action of oxytocic peptides by increasing the affinity of the receptor sites for the peptide. For example, the addition of Mg²⁺ to the medium bathing isolated mammary strips caused a parallel displacement to the left of log dose-response curves for a series of oxytocin analogues (24). The maximum response to oxytocin was unchanged by the addition of Mg²⁺ (24). Comparable results have been obtained for a number of oxytocin analogues with the isolated uterus (5) and isolated blood vessels (8, 9). Studies with other oxytocic analogues have shown that both the maximal response of the target tissue as well as the affinity for the hormone was diminished in the absence of Mg²⁺ (7, 9). It has been assumed that this reduction in response was due to a decrease in the intrinsic activity of the peptides in the absence of Mg²⁺ (7, 9). However, it is equally possible that the reduced response was the result of a reduction in the concentration of available receptor sites for the oxytocic peptides. Therefore, Mg²⁺ may affect both the affinity and concentration of oxytocin receptor sites in intact, isolated target organs as well as in particulate fractions from the mammary gland. Our observations on the comparative activities of Mn²⁺ and Mg²⁺ are consistent with results showing that 0.1 mM Mn²⁺ was more effective than 0.5 mM Mg²⁺ in potentiating the potency of several oxytocin analogues in the isolated uterus (5). The order of effectiveness of divalent metal ions in increasing the affinity of the mammary receptor sites for oxytocin was Ca²⁺ > Mn²⁺ > Ni²⁺ > Mg²⁺ > Zn²⁺. No specific binding occurred in the presence of Ca²⁺, Cu²⁺, and Fe²⁺. This order is the same as that reported by Schild (27) for the effect of metal ions on lysine vasopressin activity on the depolarized uterus of the rat.

There is no clear relationship between the relative activities of the metal ions and size as estimated by their crystal ionic radii and the strength of their coordination complexes (28). The order of magnitude of the equilibrium constants for complexation of divalent metal ions with nitrogen donor ligands (Irving-Williams series) is Ca = Mg < Mn < Co < Ni < Cu < Zn (29). Although there is some correlation between the rank order of cobalt, manganese, and magnesium and their effectiveness in promoting oxytocin-receptor binding, it is probably inappropriate to generalize from simple complexes to complicated systems. Multiple binding sites may be arranged in specific configurations which we can neither examine nor predict in the system under study. Although the present studies do not delineate the precise mechanisms by which metal ions affect the activity of oxytocin, they provide a model that serves to explain the role of metal ions in potentiating oxytocin action.

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REFERENCES
1. van Dyke, H. B., and Hastings, A. B. (1928) Am. J. Physiol. 85, 563-577
2. Munsick, R. A., Sawyer, W. H., and van Dyke, H. B. (1960) Endocrinology 66, 860-871
3. Clegg, P. C., Hopkinson, P., and Pickles, V. R. (1963) J. Physiol. (Lond.) 187, 1-17
4. Munsick, R. A., and Jeronimus, S. C. (1965) Endocrinology 76, 90-96
5. Bentley, P. J. (1965) J. Endocrinol. 23, 215-222
6. Krejci, I., and Polacek, I. (1968) Eur. J. Pharmacol. 2, 393-398
7. Walter, R. Dubois B. M., and Schwartz, I. L. (1968) Endocrinology 83, 979-985
8. Soonlyo, A. V., Woo, C. Y., and Soonlyo, A. P. (1966) Am. J. Physiol. 210, 705-714
9. Altura, B. M. (1975) Am. J. Physiol. 228, 1615-1620
10. Soloff, M. S., and Swartz, T. L. (1973) J. Biol. Chem. 248, 6471-6478
11. Soloff, M. S. (1976) in Hormone-Receptor Interaction: Molecular Aspects (Levey, G. S., ed) pp. 129-151, Marcel Dekker, Inc., New York
12. Soloff, M. S., Schroeder, B. T., Chakraborty, J., and Pearlmutter, A. F. (1977) Fed. Proc. 36, 1861-1866
13. Soloff, M. S. (1976) in Methods in Receptor Research (Blecher, M. ed) pp. 511-531, Marcel Dekker, Inc., New York
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
15. Hanlon, D. P., Watt, D. S., and Westhead, E. W. (1966) Anal. Biochem. 18, 225-233
16. Hall, J. L., Swisher, J. A., Braun, D. G., and Liden, T. M. (1962) Inorg. Chem. 1, 409-413
17. Bai, K. S., and Martell, A. E. (1969) J. Inorg. Nucl. Chem. 31, 1697-1707
18. Schroeder, B. T., Chakraborty, J., and Soloff, M. S. (1977) J. Cell Biol. 74, 428-440
19. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660-672
20. Hill, A. V. (1910) J. Physiol. (Lond.) 40, iv-vi
21. Silhavy, T. J., Szemclman, S., Boos, W., and Schwartz, M. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2120-2124
22. Strickland, S., Palmer, G., and Massey, V. (1975) J. Biol. Chem. 250, 4048-4052
23. Campbell, B. J., Chu, F. S., and Hubbard, S. (1963) Biochemistry 2, 764-769
24. Polacek, I., and Krejci, I. (1969) Eur. J. Pharmacol. 7, 85-88
25. Moore, R. D., and Zarrow, M. X. (1965) Acta Endocrinol. 48, 186-196
26. Eigen, M. (1963) Pure Appl. Chem. 6, 97-115
27. Schild, O. (1971) Ciba Found. Study Group 39, 119-138
28. East, R. C., Selby, S. M., and Hodgman, C. O., eds (1964) Handbook of Chemistry and Physics, 45th Ed, p F-89, The Chemical Rubber Co., Cleveland
29. Cotton, F. A., and Wilkinson, G. (1962) Advanced Inorganic Chemistry, p. 589, Interscience Publishers, New York
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