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Oxytocin Action: Lack of Correlation Between Receptor Number and Tissue Responsiveness

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Brattleboro rats exhibit diabetes insipidus (DI) because of a genetic autosomal recessive defect in the synthesis of vasopressin; oxytocin is synthesized normally. Preliminary work suggests that elevated circulating oxytocin levels may compensate for the absence of vasopressin. To evaluate the consequences of presumed elevations of oxytocin levels, oxytocin binding and tissue responsiveness have been measured in the uterus and epididymal fat cells of homozygous-DI (HoDI) and heterozygous-DI (HeDI) animals and Sprague-Dawley and Long-Evans controls. Surprisingly, whereas membranes from HoDI rat uteri exhibited an 85% reduction in oxytocin binding, the biological response (contraction) to oxytocin was indistinguishable from the uteri of HeDI or Sprague-Dawley animals. The uterine response to carbachol was also normal in HoDI rats. In contrast, in adipocytes from HoDI animals, the biological response to oxytocin (glucose oxidation) was abolished, whereas the binding of oxytocin was normal; insulin-stimulated glucose oxidation was, however, normal. These results indicate that receptor binding, while critical to hormone action, is not the sole determining factor. With oxytocin action, postreceptor mechanisms are most important in determining oxytocin responsiveness.

Key words: oxytocin receptors, diabetes insipidus, Brattleboro rats, oxytocin resistance, glucose oxidation, uterine contraction, postreceptor mechanisms

It is now well established that responsiveness of a tissue to a hormone is governed not only by the concentration of circulating hormone, but also by the concentration of receptors and the affinity receptors demonstrate for a hormone [1–4]. However, much less is known about the factors that modulate the coupling of receptor occupation to tissue response. In this report, we describe studies of the binding and action of oxytocin in the uterus and adipocytes of the Brattleboro rat. There appears to be no correlation

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Abbreviations used: HoDI, homozygous diabetes insipidus; HeDI, heterozygous diabetes insipidus; LE, Long-Evans; KRB, Krebs-Ringer bicarbonate; BSA, bovine serum albumin; DI, diabetes insipidus.

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between receptor content and tissue response to oxytocin. In these animals, postreceptor mechanisms seem to be most important in determining oxytocin action.

Rats of the Brattleboro strain exhibit diabetes insipidus because of a recessively, inherited genetic defect in the biosynthesis of vasopressin [5–7]. Although the homozygous animals (HoDI) do not synthesize vasopressin, oxytocin is synthesized normally, and the pituitary oxytocin content is about 1/3 that of either heterozygous animals (HeDI) or control rats [6]. It has been suggested that the reduced pituitary levels, stemming from an increased rate of oxytocin secretion, would lead to elevated serum oxytocin levels [6]. Hormone levels measured by radioimmunoassay in plasma of Wistar rats and of HoDI rats agree with this suggestion; 9.4 ± 2.6 pg/ml (n = 19) in Wistar rats and 50.5 ± 15.6 pg/ml in HoDI rats (n = 8) [8]. Since it would be expected that elevated serum oxytocin levels might cause receptor "down-regulation" with consequent tissue resistance [2, 4], it was of interest to examine in the HoDI animal oxytocin receptor content and hormone responsiveness in two tissues known to respond to oxytocin (adipose tissue and uterus). We observe that, although adipocytes from HoDI animals bind the same amount of oxytocin as do control animals, the cells are unresponsive to oxytocin (increased glucose oxidation). In contrast, uterine membranes from HoDI animals possess only 1/7 the number of oxytocin receptors compared with control animals, but tissue responsiveness (contraction) is unaltered.

MATERIALS AND METHODS

The following chemicals were generously provided: [^{3}H] oxytocin (34 Ci/mmole) by Dr. M. Soloff, Medical College of Ohio (Toledo, Ohio); porcine zinc insulin (exp. 491-4) by Dr. J. Clement, Connaught Laboratories (Toronto, Canada); and synthetic oxytocin by Dr. G. Moore, University of Calgary (Calgary, Canada). Syntocinon (10 USP units/ml) was purchased from Sandoz (Dorral, Canada). Concentrations of oxytocin are expressed in molar units and are based on 1 mg oxytocin having 500 USP units of uterine contractile activity [9]. Carbachol was purchased from Sigma (St. Louis, Missouri), bovine serum albumin (fraction V, lot T13904) from Armour Pharmaceutical (Kankakee, Illinois), collagenase (4196 CLS 48M201) from Worthington Biochemical Corporation (Freehold NJ), and Honvol (synthetic diethylstilbestrol) from Horner Pharmaceutical Company (Montreal, Canada).

^{125}I-Oxytocin

25 μl oxytocin (240 μg/ml in 0.2 M acetic acid) was added to 50 μl 0.5 M phosphate buffer, pH 7.5, in a 500 μl reaction vessel. 10 μl chloramine-T (100 μg/ml) was added. This was followed immediately by the addition of 10 μl carrier-free sodium ^{125}I-iodide (New England Nuclear). Following 10 sec of light shaking 100 μl of 25% (w/v) bovine serum albumin (BSA) in 0.5 M phosphate buffer pH 7.5 was added. The reaction mixture was then washed into a tube containing Rexyn 202 resin (Fisher) equilibrated, with 0.25% acetic acid. After 10 sec of shaking, the suspension was centrifuged and the supernatant was applied to a Sephadex G-25 column (11 × 1.0 cm), equilibrated and eluted with 0.25% acetic acid [10]. One ml fractions were collected and measured for radioactivity (Searle Automatic Gamma Counter). Two radioactive peaks were eluted; the first was ^{125}I-BSA, and the second was ^{125}I-oxytocin. ^{125}I-Iodine remained adsorbed to the Sephadex column. The contents of the tube after the second peak were used in binding assays. This tube was believed to be essentially free of unlabeled oxytocin

since iodo-oxytocin elutes after oxytocin in this chromatographic system [11]; the specific activity (2,500 Ci/mg) was calculated on this premise. The absolute specific activity of ^{125}I-oxytocin was not critical in the calculation of receptor number since the radioligand was used only in trace amounts compared with unlabeled oxytocin.

Preparation of Isolated Adipocytes

Rats homozygous (HoDI) (300–400 g) and heterozygous (HeDI) (350–450 g) for diabetes insipidus and Long-Evans (LE) rats (the parent strain to the DI rats) (300 g) were killed by decapitation. Epididymal fat pads were removed, cut into small pieces, and digested at 37°C at pH 7.5 by gently shaking for 30 min in a 3 ml collagenase solution (2 mg/ml) in Krebs-Ringer bicarbonate (KRB) buffer containing 2% BSA and equilibrated with 95% O_2/5% CO_2. Collagenase digestion was followed by isolation of adipocytes as previously described [12].

Glucose Oxidation

The oxidation of U-^{14}C -glucose to $^{14}CO_2$ was measured by the method of Rodbell [13]. Each assay bottle contained 0.2 ml of the cell suspension, 0.1 ml of insulin (0–200 ng/ml) or oxytocin (0–2,500 nM), and 1.7 ml of a substrate solution containing 0.28 mM of glucose and 0.2 μCi of U^{14}C -glucose in KRB-2% BSA. Cells were incubated at 37°C for 1 h. Filter paper (1 × 15 cm) in hanging wells was treated with 0.2 ml hyamine hydroxide (Packard), and the vessel contents were acidified with 0.3 ml 2N sulphuric acid. After allowing 30 min at room temperature for $^{14}CO_2$ to be adsorbed by the base-treated filter paper, the filter paper was removed, placed in 15 ml liquid scintillation fluid (ACS, Amersham), and radioactivity was measured (Beckman LS-250). Efficiency of the detection and scintillation system was 40–60%.

Oxytocin Binding to Fat Cells

Each assay tube contained 50 μl of ^{125}I -oxytocin (50,000 cpm), 50 μl of unlabeled oxytocin (0 to 4.5 μM), and 0.4 ml of fat cell suspension (0.6–1.2 × 10^5 cells). All solutions were made up in KRB-2% BSA. Assay tubes were gently agitated at 37°C for 15 min. Two 0.2 ml aliquots were removed and centrifuged over 0.1 ml dinonyl phthalate in 0.4 ml microfuge tubes as described by Gameltoft and Gliemann [14]. The upper halves of the tubes containing the fat cell layer were collected in 12 × 75 mm Pyrex tubes, and radioactivity was measured. Radioactivity in aliquots from the aqueous phase was also measured to determine unbound hormone. Nonspecific binding was estimated as that amount of ^{125}I -oxytocin bound in the presence of 4.5 μM oxytocin, a concentration 1,000 times greater than the affinity constant of the receptor for oxytocin as measured by bioassay [15].

Adipocyte Count

The number of fat cells in all experiments was estimated from solution triglyceride content [16]. Adipocyte suspensions were diluted 1:10 with KRB-2% BSA buffer. Of the diluted fat cell suspension, 0.1 ml was used in the Pierce Triglyceride Rapid Stat Kit (Pierce C-37). The reagent volumes in the latter procedure were appropriately halved and centrifugation steps deleted. With the aid of a calibration curve relating epididymal fat pad cell size to rat size [17], a cell number could be calculated from the triglyceride content.

Assay of Uterine Response

Dissected uterine horns from estrogenized animals (50 μg diethylstilbestrol in 50 μl H_2O, intramuscularly, 24 h prior to bioassay) were cut into 1.5 cm lengths and mounted under 1 g tension in 4 ml of oxygenated Mg-free bathing solution (pH 7.0; NaCl, 154 mM; $NaHCO_3$, 1.8 mm; KCl, 5.6 mM; glucose, 1.4 mM; $CaCl_2$, 0.18 mM) [18]. Contraction (isometric) in response to increasing concentrations of oxytocin and carbachol was measured with a Statham force-displacement transducer. Each uterus was tested with each concentration of both agents, the sequence of drugs varying. Between each dose, the tissue was washed once and allowed 5 min to reach basal tension. Between the highest dose of one compound and the next drug solution tested, the tissue was washed three times and allowed 30 min to reach basal tension. Contraction is reported as percent of maximal response.

Preparation of 20,000g Uterine Particulate Fraction

Female Sprague-Dawley rats (200–250 g) and female homozygous Brattleboro DI rats (175–225 g) were injected intramuscularly with 50 μg of diethylstilbestrol in 50 μl distilled water 16 to 24 h before decapitation. Uterine horns were removed and homogenized in nine volumes of Tyrode's solution [19] with a Brinkman Polytron PT homogenizer for three bursts of 15 sec at 10,000 rpm at 4°C. All subsequent operations were at 4°C. The homogenate was centrifuged at 1,000g for 10 min and the resulting supernatant was centrifuged at 20,000g for 10 min. The pellet was resuspended in Tyrode's solution and recentrifuged at 20,000g. The pellet thus obtained was resuspended and stored at −60°C [20].

Oxytocin Binding to Uterus

Frozen particulate fractions of the uterus were thawed slowly, centrifuged, and then resuspended in Tris-maleate buffer at 4°C (containing 5 mM magnesium chloride, pH 7.6). Two hundred μl of particulate suspension (0.6–1 mg protein) was added to varying amounts of [^{3}H] oxytocin (6,000 dpm to 30,000 dpm) and unlabeled oxytocin in Tris-maleate buffer in polyallomer tubes to a final volume of 250 μl. The binding mixture was incubated at 20°C with shaking for 1 h [20]. The reaction was terminated by centrifugation of the mixture at 20,000g for 10 min at 4°C. The pellet was suspended with 1 ml Tris-maleate buffer and then dissolved by addition of 100 μl sodium hydroxide and warming at 60°C for 30 min. The solution was then placed on Whatman filter paper (grade 3, 2.3 cm diameter). The filter paper was compacted in a pill press (Parr Instrument Company), oxidized in a Packard Tri-carb Model 306 Oxidizer, dissolved in Monophase-40 scintillation fluid (Packard), and the radioactivity was determined [20].

A binding procedure similar to the fat cell binding assay was also investigated. In this procedure, 400 μl of particulate fraction was added to 25 μl ^{3}H-oxytocin (16,000 dpm), 25 μl Tris-maleate buffer, and 50 μl unlabeled oxytocin (0 to 4,500 nM). Following incubation at 20°C for 60 min, 200 μl aliquots were placed in 400 μl microfuge tubes and centrifuged in a Beckman Microfuge B for 10 min (at 4°C). The supernatant was aspirated off and the pellet suspended by a brief centrifugation in 100 μl dibutyl phthalate. The pellet was removed with a Pasteur pipette and placed in ACS (Amersham) or Bray's liquid (New England Nuclear) scintillation fluid for liquid scintillation counting. This method yielded results equivalent to the data obtained by the procedure described above. However, nonspecific binding proved to be higher, and the method was not chosen for routine studies.

Radioactivity bound to the 20,000g particulate fraction in the presence of 10 μM of unlabeled oxytocin was assumed to be nonspecifically bound radioligand and was subtracted from the protein-bound radioactivity found in all binding assay tubes. Binding data was analyzed by the method of Scatchard [21].

Protein concentrations were determined by the method of Lowry et al [22] using bovine serum albumin in preparation of the calibration curve.

RESULTS

Stimulation of Glucose Oxidation by Oxytocin

Glucose oxidation in adipocytes from LE and HeDI rats was stimulated by oxytocin (Fig. 1). In HeDI animals, half-maximum stimulation occurred at approximately 5 nM oxytocin; this value can be compared with an estimated K_d of about 5 nM for oxytocin binding (see below). In contrast, fat cells from HoDI rats did not respond at all to oxytocin (stimulation of glucose oxidation, Fig. 1C, lower panel), whereas the response to insulin was equivalent to that of adipocytes from HeDI or LE animals.

^{125}I-Oxytocin Binding to Adipocytes

Monoiodo-oxytocin has been found to stimulate glucose oxidation in isolated rat adipocytes. The maximal effect of I-oxytocin was 75–80% that of oxytocin [11]. Preliminary studies in this laboratory demonstrated that fat cell binding of ^{125}I-oxytocin was time dependent (optimal after 15 min at 37°C), and that once the radioligand was bound, it could be displaced by unlabeled oxytocin (data not shown [11]). Based on these observations, we have assumed that measurement of ^{125}I-oxytocin binding is a measurement of oxytocin receptors. Our binding data with adipocytes from both HoDI and HeDI Brattleboro rats indicate two classes of binding sites (Fig. 2). The dissociation constants and number of oxytocin receptors may be estimated for the high affinity binding site from the insert of Figure 2: HoDI and HeDI Brattleboro rat adipocytes have approximately 5×10^4 oxytocin binding sites with K_d approximately 5 nM (Table I). These results are in agreement with the measurements of Bonne and Cohen [15] ($K_d \cong 5$ nM; 3×10^4 receptors/cell), who used tritiated rather than iodinated oxytocin for binding measurements. The low-affinity oxytocin binding sites appear to have the same affinity in both HoDI and HeDI rats, but there appear to be a greater number of these sites in HoDI rats (Fig. 2).

Stimulation of Uterine Contraction by Oxytocin

Figure 3 illustrates that the uteri from Sprague-Dawley, HeDI, and HoDI rats previously injected with diethylstilbestrol exhibited identical dose-response curves. In addition to carbachol, angiotensin II and arginine vasopressin (data not shown) were tested. These agents were unaltered in their ability to stimulate uterine contraction irrespective of the source of the tissue.

^{3}H-Oxytocin Binding to the 20,000g Particulate Fraction of Rat Uteri

Figure 4 illustrates the Scatchard analysis [21] of the uterine oxytocin binding data. Uterine membranes from Sprague-Dawley rats demonstrated a K_d of 2.2 nM and bound approximately 170 fmole of oxytocin per mg protein. These results are in agreement with the measurements of Soloff and Pearlmutter [23], who found a K_d of 1.8 nM and 180 fmole oxytocin bound per mg protein. The same particulate fraction from the

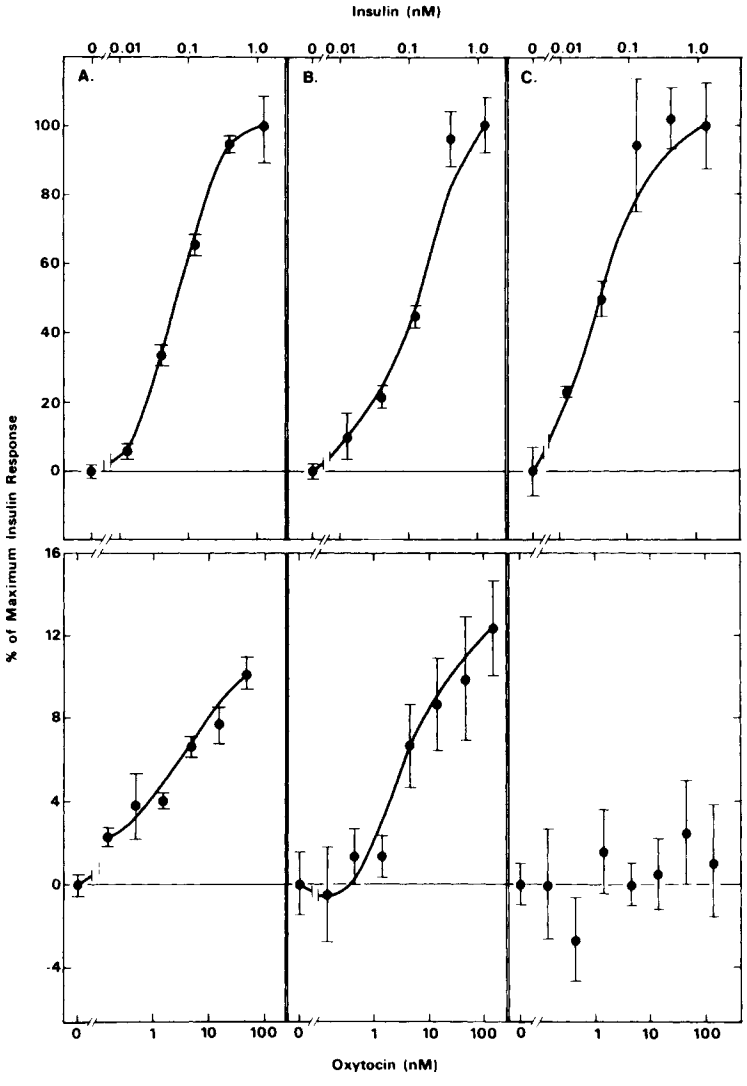

Fig. 1. Stimulation of glucose oxidation in epididymal adipocytes by insulin or oxytocin; A) Upper and lower: Long-Evans rats, B) upper and lower: HeDI Brattleboro rats, C) upper and lower: HoDI Brattleboro rats. The results are expressed as a percentage of maximal insulin stimulation above the basal rate of glucose oxidation. Upper panels: insulin stimulation. Lower panels: oxytocin stimulation. The vertical bars through the data points indicate the standard error of the mean for 3 to 9 separate experiments.

homozygous DI Brattleboro rat, however, demonstrated quite different binding properties for oxytocin: K_d of 1 nM and only 25 fmole oxytocin bound per mg protein (Fig. 4, Table I).

In addition to the high affinity binding site for oxytocin on rat uteri, there also appears to be a low-affinity site; this site is demonstrated by the nearly horizontal portion of the Scatchard analyses (Fig. 4). Low-affinity sites have not been described before, but they may be a consequence of differences in membrane preparation between our laboratory and others.

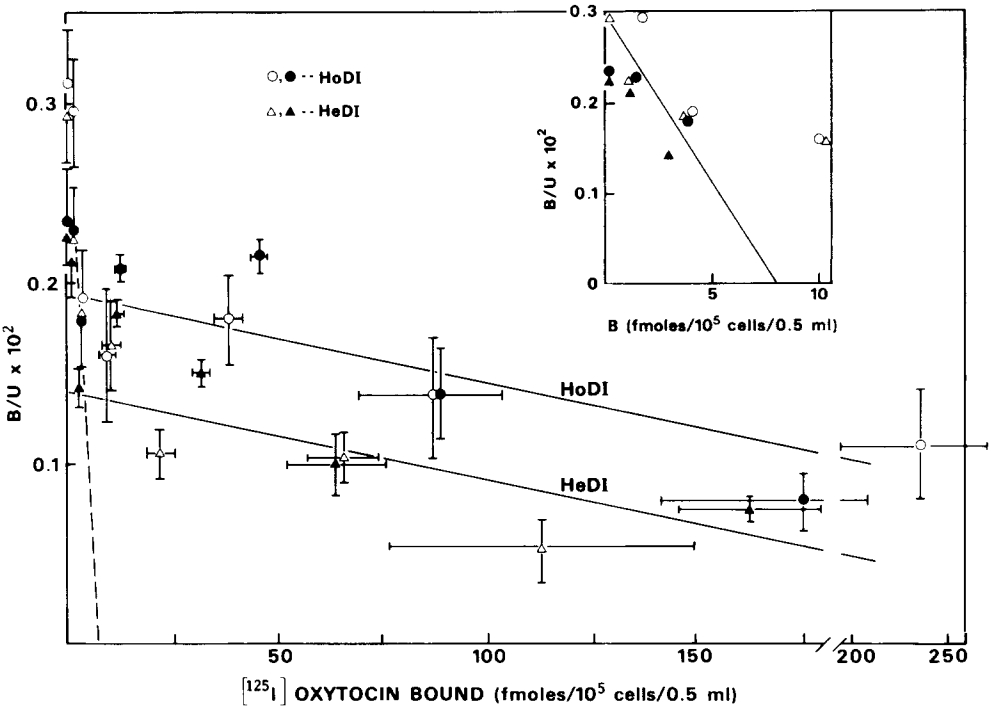

Fig. 2. [^{125}I]-Oxytocin binding to adipocytes. Insert: Scatchard [20] analysis of binding data for 0 to 10 nM unlabeled oxytocin. Adipocytes were obtained from HeDI Brattleboro rats (△,▲) or HoDI Brattleboro rats (○, ●). Symbols are the mean, and the vertical and horizontal bars represent the standard mean error (n = 4).

TABLE I. Tissue Affinity and Receptor Number for Oxytocin

Receptor preparation	K_d[a] (nM)	Receptor number[a]
Adipocyte		
Heterozygous DI Brattleboro rat Homozygous DI Brattleboro rat	5	5×10^4/cell
Uterine (20,000g) particulate fraction		
Sprague-Dawley rat	2.2	170 fmole/mg protein
Homozygous DI Brattleboro rat	1.0	25 fmole/mg protein

[a]High affinity sites.

DISCUSSION

The main finding of our study is that, in the HoDI rat, oxytocin responsiveness in adipocytes is abolished, despite the binding of normal amounts of oxytocin. However, the uterine response is normal in the face of an 85% reduction in oxytocin receptor content. Our observations with the uterine tissue can be contrasted with the effects of magnesium ion, which increases the number of oxytocin binding sites in uterine tissue [20] without affecting the tissue response to oxytocin [18]. Thus, in the two target

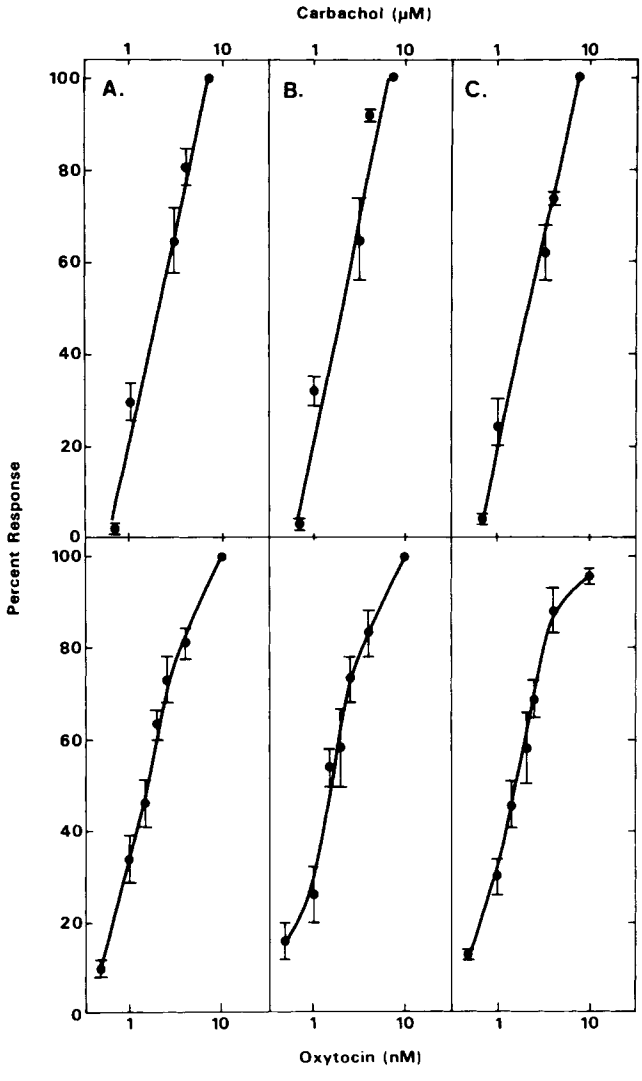

Fig. 3. Uterine dose-response curves for oxytocin and carbachol. Sprague-Dawley (A), HeDI (B), and HoDI (C) rats were injected with diethylstilbestrol 24 h prior to removal of uterine tissue. Symbols denote the mean, and the vertical bars indicate the standard error of measurment where the number of uteri (n) were: n = 5 (B) or 6 (A, C) for carbachol and n = 15 (A), 5 (B), or 12 (C) for oxytocin.

tissues selected for study, there is a lack of correlation between receptor content and tissue responsiveness. Clearly, the widely held belief that receptor down-regulation can, in general, lead to reduced tissue sensitivity [4] must be viewed with caution.

Our observations underline the importance of postreceptor mechanisms that govern oxytocin action. In the uterus of the HoDI animal, it would appear that the efficiency of coupling of receptor occupation to contraction is improved, compared with control animals. In contrast, in the adipocytes, the homozygous animals appear to have inherited a postreceptor defect specific for oxytocin, since insulin responsiveness is un-

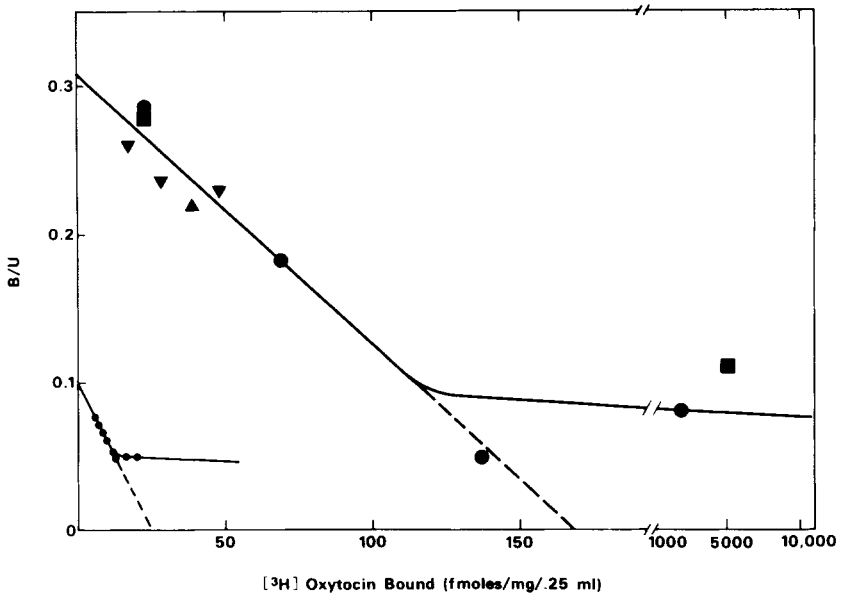

Fig. 4. ^{3}H -Oxytocin binding to rat uterus membranes. Large symbols represent binding data to the uterine membranes from Sprague-Dawley rats. (●, ■) Samples contained 25 μl Tris-maleate buffer, 50 μl unlabeled oxytocin (0–4.5 μM), and 0.4 ml membrane preparation (1.13 mg protein/ml). Bound and unbound [^{3}H]-oxytocin was separated by centrifugation in a Microfuge B at 22°C (■) or at 4°C (●). (▲) Binding tubes contained 25 μl ^{3}H -oxytocin, 25 μl buffer or unlabeled oxytocin (4.5 μM), and 200 μl membrane preparation (4.4 mg protein/ml). (▼) Binding tubes contained 10, 20, or 30 μl ^{3}H -oxytocin, 200 μl membrane preparation (4 mg protein/ml), and Tris-maleate buffer to a total volume of 250 μl. The smaller symbol (●) represents binding data to the uterine fraction from HoDI Brattleboro rats. Binding tubes contained 10, 12, 15, 20, 30 (2X), 40, or 50 μl ^{3}H -oxytocin, 200 μl membrane preparation (5 mg protein/ml) and Tris-maleate buffer to a total volume of 250μl. Data plotted are the means of n = 2 except for ● where n = 4. Except for those experiments designated by ● and ■, bound and unbound ^{3}H -oxytocin was separated and protein bound radioactivity was determined by previously described methods [20].

altered. The adipocyte response to oxytocin was absent both in estrogenized (data not shown) and untreated animals. It will be important to determine whether this defect is due to an inherited alteration in the adipocyte oxytocin receptor that affects receptor-effector coupling or whether the defect is a consequence of the hypothalamic endocrine abnormality present in the HoDI animals. This latter possibility can be evaluated by hormone replacement therapy in the HoDI animal, which is known, for example, to respond to vasopressin [24].

The premise that prompted our study was that plasma oxytocin levels elevated in HoDI animals [8], may lead to changes in tissue receptor content. The reduced receptor content of the uterus is in concert with our expectations of raised serum oxytocin levels; however, the unaltered binding of oxytocin in the adipocytes is not in keeping with our supposition.

Since estradiol is known to increase the uterine oxytocin receptor content [22, 24], our study, done with estrogenized animals, was performed under conditions optimized for receptor content and response. Thus, although the uterine response in stilbestrol-treated HoDI animals was normal despite a marked reduction in oxytocin receptors, it is possible that in nonestrogenized or ovariectomized animals, the receptor content might be reduced further to a level that would compromise tissue responsiveness. From our studies, it is evident that the Brattleboro rat provides an attractive model system in which one can study a number of interesting questions related to receptor content and tissue responsiveness.

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