[^{3}H]-[Thr^4,Gly^7]OT: a highly selective ligand for central and peripheral OT receptors

JACK ELANDS, CLAUDE BARBERIS, AND SERGE JARD
Centre National de la Recherche Scientifique-Institut National de la Sante et de la Recherche Medicale de Pharmacologie-Endocrinologie, 34094 Montpellier Cedex, France

ELANDS, JACK, CLAUDE BARBERIS, AND SERGE JARD. *[^{3}H]-[Thr^4,Gly^7]OT: a highly selective ligand for central and peripheral OT receptors.* Am. J. Physiol. 254 (Endocrinol. Metab. 17): E31–E38, 1988.—Oxytocin receptors in rat hippocampal synaptic plasma membranes were compared with mammary gland and uterine oxytocin receptors. For this purpose, a highly specific oxytocic agonist [Thr^4,Gly^7]oxytocin was tritiated. We demonstrated that this ligand labels oxytocin receptors selectively. Scatchard analyses revealed a high affinity for all the oxytocin receptors investigated, with equilibrium dissociation constants between 1.0 and 2.0 nM. Binding appeared to take place at a single population of receptor sites. Competition experiments confirmed the high affinity of arginine vasopressin for hippocampal oxytocin receptors but also revealed that mammary gland and uterine oxytocin receptors do not discriminate more efficiently between oxytocin and arginine vasopressin. This lack in specificity is not affected by applying different concentrations of Mg ions.

rat; hippocampus; uterus; mammary gland; oxytocin structural analogues; oxytocin antagonists

OXYTOCIN (OT) is recognized not only as a hormone but also as a neuropeptide. It is synthesized in hypothalamic magnocellular neurons and transported to the neurohypophysis to be released into the general circulation to reach its peripheral targets. Hypothalamic OT-producing magnocellular neurons also project to the olfactory, amygdaloid, and hippocampal areas (33). At the periphery, the major biological functions of OT are its uterotonic and galactobolic activities, both reflecting OT-induced contraction of smooth muscle cells in the uterine myometrium and myoepithelial cells in the mammary gland. OT also exerts an insulin-like activity on adipocytes (16).

Tritiated OT has been used to demonstrate OT receptors among others in uteri of rat, sow (30), and human (13, 15), in rat-lactating mammary gland (29), in rat adipocytes (4), and in porcine male genital tract (19). Rat and rabbit uterine OT receptor concentrations are enhanced by estrogen and diminished by progesterone (14, 23).

Tritiated OT has also been used in autoradiographical approaches to localize OT binding sites in brain tissue. Relatively high concentrations of OT binding sites were detected in the rat olfactory nucleus, central amygdala, hypothalamic ventromedial nucleus, ventral subiculum of the hippocampus, and posterior pituitary (6, 8, 32). The number of OT binding sites in the ventromedial nucleus of the hypothalamus and, to a lesser extent, in the central amygdala was shown to be increased by estrogen (9) and decreased by castration (31).

Examination of the ligand selectivity of the specific OT binding sites in the hippocampus revealed that these sites do not discriminate efficiently between OT, arginine vasopressin (AVP), and arginine vasotocin (AVT) (2, 12). The unexpected high affinity of AVP and AVT for this presumed OT receptor led to the conclusion that hippocampal sites might be different from the known peripheral OT receptors (2).

This study was set up to characterize and compare hippocampal, uterine, and mammary gland OT receptors under identical experimental conditions, using the same ligands. To improve specificity of the characterization a novel, more selective, oxytocic ligand was introduced. A high specificity is of importance when AVP and OT receptors are colocalized, as in the rat hippocampus. The selectivity of a ligand can be expressed by its affinity index for the receptors involved. This affinity index is calculated as the ratio of affinities of one ligand for two different receptor types. In the hippocampus the AVP-receptor-OT-receptor affinity index is 14.3 for OT and 0.4 for AVP, while it is >6,000 for OH-[Thr^4,Gly^7]oxytocin (2). A closely related analogue, [Thr^4,Gly^7]oxytocin, with essentially the same properties, was tritiated. This highly active oxytocic agonist, without apparent vasopressor or antidiuretic activity (18), with a high affinity for hippocampal OT receptors, and a very low affinity for central vasopressin (2), peripheral V_{1a} vasopressin (Elands and Barberis, unpublished observations) and V_2 vasopressin receptors (Cantau et al., personal communication) made it possible to investigate the OT receptor specifically.

MATERIALS AND METHODS

Preparation of [^{3}H]-[Thr^4,Gly^7]OT. The OT analogue, [4-threonine,7-glycine]oxytocin ([Thr^4,Gly^7]OT) was tritiated on the tyrosyl residue in *position 2* according to Pradelles et al. (24). Tritiation of the peptide was performed by Dr. J. L. Morgat, to whom we are greatly indebted. The labeled peptide was purified by high-pressure liquid chromatography (HPLC) on a Waters reversed phase C_{18} μ-Bondapak column (Fig. 1). Elution at 1 ml/min was performed with a linear (40 min) gradient of 10–50% *solvent B* in *solvent A*. *Solvent A* was 0.1%

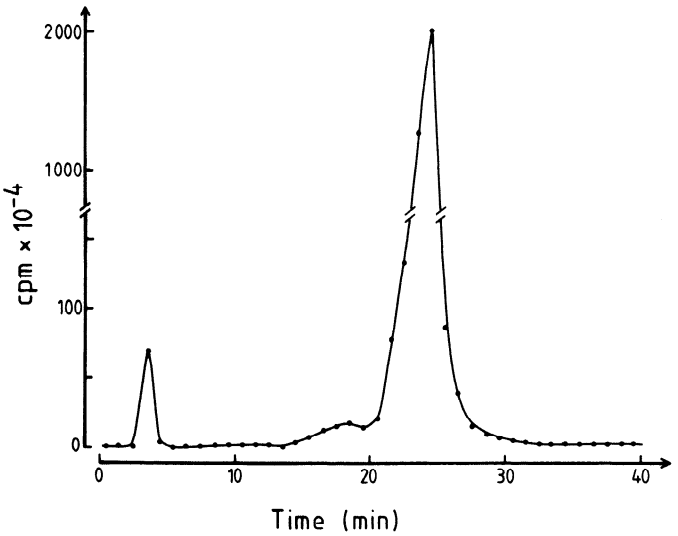

FIG. 1. High-pressure liquid chromatography elution profile of [^{3}H]-[Thr^4,Gly^7]OT purification on a Waters reversed phase C_{18} μBondapak column.

trifluoroacetic acid (pH 3.5), *solvent B* was 0.1% trifluoroacetic acid (pH 3.5) in 75% acetonitrile. [^{3}H]-[Thr^4,Gly^7]OT was further purified by affinity chromatography, using neurophysin bound to Sepharose-4B (7). Its specific radioactivity was 23.3 Ci/mmol.

Materials. [^{3}H]AVP (sp act = 64.7–70.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Unlabeled [Thr^4,Gly^7]OT, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)-2-D-phenylalanine,4-isoleucine]arginine vasopressin {d(CH_2)$_5$[D-Phe^2,Ile^4]AVP} and 9-desglycynamide-[1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)-2-*O*-methyltyrosine,4-threonine,8-ornithine]vasotocin {desGly(NH_2)^{9}d(CH_2)$_5$-[Tyr(Me)^{2}Thr^4]OVT} were kindly donated by Professor M. Manning (Toledo, OH). AVP, AVT, and OT were purchased from Bachem (Bubendorf, Switzerland). The biological activities of all peptides used are indicated in Table 1. Sucrose and $MgCl_2$ were purchased from Merck (Darmstadt, FRG) and Tris from Sigma Chemical (St. Louis, MO).

Membrane preparation. Female Wistar rats were purchased from Iffa Credo (Lyon, France). Hippocampal membranes were prepared from the ventral part of the hippocampus, according to a previously described procedure (2, 3). Briefly, ventral hippocampi from 40 rat brains were dissected and immersed in ice-cold 0.32 M sucrose. After homogenization with a Potter homogenizer equipped with a perspex pestle (clearance 0.25 mm), the homogenate was centrifuged at 1,000 *g* for 20 min. The supernatant was spun down at 11,000 *g* for 20 min. The pellet was submitted to an osmotic shock in 5 mM Tris·HCl (pH 8.1) for 30 min at 0°C. A synaptic membrane fraction was prepared by density gradient centrifugation (34, 28.5 and 10% sucrose, wt/vol) for 110 min at 60,000 *g* in a SW-28 swing-out bucket rotor (Beckman ultracentrifuge). Membranes were collected at the 28.5–34% interface, dispersed in 50 mM Tris·HCl (pH 7.4) and 10 mM $MgCl_2$, washed, and resuspended in the same medium.

For the preparation of myometrial membranes uteri were taken from estrogenized or ovariectomized (OVX) rats. Estrogenized rats received subcutaneously 10 $\mu g \cdot 100$ g body $wt^{-1} \cdot day^{-1}$ 17β-estradiol benzoate in oil vehicle during 7 days. Ovariectomy was performed 7 days before the experiment, and the animals were treated with oil vehicle. Uteri were removed and scraped free of endometrium. The tissue was homogenized in ice-cold 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 300 mM KCl buffer with a Polytron (Kinematica, Kriens, Switzerland) at setting six for three periods of 10 s. The homogenate was spun down at 500 *g* for 10 min. The resulting supernatant was centrifuged at 12,000 *g* for 30 min. The pellet was washed in 10 mM Tris·HCl (pH 7.4) and 1 mM EDTA and resuspended in 15 ml 10% sucrose (wt/vol), 10 mM Tris·HCl, and 1 mM EDTA that was layered onto 15 ml 35% sucrose (wt/vol), 10 mM Tris·HCl, and 1 mM EDTA. After centrifugation for 2 h at 100,000 *g* in a SW-28 swing-out bucket rotor (Beckman ultracentrifuge), the membranes were collected at the 10–35% interface. The membranes were dispersed in 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, washed, and resuspended in the same medium.

Mammary glands were obtained from 14- to 18-day lactating rats. Dissected mammary gland was freed of fascia and connective tissue and homogenized as de-

TABLE 1. *Biological activities of OT, AVP, and some of their analogues*

Peptide	Biological Activities			
	Vasopressor	Antidiuretic	Mammary gland milk ejection	Uterus contraction
AVP	369±6[a]	323±16[a]	69±9[bf]	26[c]
AVT	243±15[b]	248±35[b]	210[bf]	246[c]
OT	4.3±0.12	4.0±0.8	474±16[f]	486±15
[Thr^4,Gly^7]OT	<0.01	0.002±0.0004	802±23	857±26
d(CH_2)$_5$[D-Phe^2,Ile^4]AVP	7.86±0.05[d]	8.24±0.08[d]		7.11±0.11[d]
desGly(NH_2)^{9}d(CH_2)$_5$[Tyr (Me)^{2}Thr^4]OVT	6.48±0.08[e]	5.3[e]		7.69±0.07[e]

Values are taken from [a] Sawyer et al. (24), [b] Berde and Boissonnas (5), [c] Mühlethaler et al. (20), [d] Manning et al. (19), [e] Sawyer et al. (unpublished results), and others from Lowbridge et al. (18). Values are expressed in U/mg. Oxytocic (OT) endocrine activities of agonists were tested at 0.5 mM Mg^{2+}; potencies of antagonists were determined in vivo. Values from references [d] and [e] are expressed in pA_2. pA_2 is the negative log of A_2, which is the concentration of agonist reducing the response to 2× units of oxytocin to equal the response to 1× unit administered before the antagonist. [f] Obtained in rabbit mammary gland, others in rat tissue. AVP, arginine vasopressin; AVT, arginine vasotocin; d(CH_2)$_5$[D-Phe^2,Ile^4]AVP, 1-(β-mercapto-β-β-cyclopentamethylenepropionic acid)-2-D-phenylalanine 4-isoleucine]arginine vasopressin; desGly(NH_2)^{9}d-(CH_2)$_5$[Tyr(Me)^{2}Thr^4]OVT, 9-desglycynamide-[1-(β-mercapto-β,β-cyclopentamethylenepropionic acid)-2-*O*-methyltyrosine,4-threonine,8-ornithine]vasotocin.

scribed above for the uterus membrane preparation in ice-cold 10 mM Tris·HCl (pH 7.4) and 1 mM EDTA. The homogenate was centrifuged for 10 min at 1,000 *g*. The resulting supernatant was centrifuged for 30 min at 12,000 *g*. The pellet was homogenized in 10% sucrose (wt/vol), 10 mM Tris·HCl (pH 7.4), and 1 mM EDTA. After this step the same procedure as for the preparation of uterus membranes was used. All experiments were done with freshly prepared membranes.

Binding assays. Membranes (85–155 μg/assay for hippocampus, 10–16 μg/assay for uterus, and 93–168 μg/assay for mammary gland) were incubated during 30 min at 30°C in 200 μl 50 mM Tris·HCl (pH 7.4), containing 10 mM $MgCl_2$ (unless stated otherwise), 1 mg/ml bovine serum albumin, and various concentrations of [^{3}H]-[Thr^4,Gly^7]OT, [^{3}H]AVP, and unlabeled peptides.

Nonspecific [^{3}H]-[Thr^4,Gly^7]OT and [^{3}H]AVP binding were determined in the presence of a 250-fold excess of OT and AVP, respectively. It was checked that determination of nonspecific [^{3}H]-[Thr^4,Gly^7]OT binding in the presence of unlabeled OT or [Thr^4,Gly^7]OT gave similar values. Bound radioactivity was separated from free radioactivity by filtration through Gelman GA-3 (Metricel, 1.2 μm) filters as described (3).

Preliminary experiments indicated that specific [^{3}H]-[Thr^4,Gly^7]OT binding to uterus and hippocampal membranes was time dependent. At a [^{3}H]-[Thr^4,Gly^7]OT concentration of 1 nM specific binding reached an equilibrium value within 20 min with a half-time of ~2.5 min and was stable for ~60 min. It was also checked that [^{3}H]-[Thr^4,Gly^7]OT was not degraded during incubation in the presence of membranes. For this purpose, [^{3}H]-[Thr^4,Gly^7]OT (2 nM) was incubated with hippocampal membranes in a final volume of 200 μl for 30 min at 30°C. After filtration (Gelman GA-3) the filtrate containing [^{3}H]-[Thr^4,Gly^7]OT was immediately analyzed by HPLC as described above. There was no detectable degradation of the labeled peptide.

The binding constants for the unlabeled peptides were determined in competition experiments in the presence of 1.7–2.0 nM [^{3}H]-[Thr^4,Gly^7]OT or 2.0 nM [^{3}H]AVP. Inhibition constants ± SD and slopes of displacement curves were calculated as described in Table 3. Dissociation constants and maximal binding capacities ± SD of concentration dependency experiments were estimated from a linear regression of the Scatchard plot analyses.

Homologues and heterologues displacement of [^{3}H]-[Thr^4,Gly^7]OT and [^{3}H]AVP binding were analyzed simultaneously using the computer program LIGAND (21). Selection between a one- and two-site model was based on an *F* test (21).

RESULTS

Kinetics of [^{3}H]-[Thr^4,Gly^7]OT binding. Specific [^{3}H]-[Thr^4,Gly^7]OT binding sites could be detected on membranes from rat hippocampus, uterus, and mammary gland. At a [^{3}H]-[Thr^4,Gly^7]OT concentration of 2 nM, which was close to the K_d value (see below), the nonspecific component of binding contributed to 52 ± 5% ($n = 5$) for hippocampal, 7 ± 2% ($n = 4$) for mammary gland, 10 ± 6% ($n = 6$) for estrogenized myometrial, and 37 ± 10% ($n = 4$) for OVX myometrial membranes.

Scatchard analysis of [^{3}H]-[Thr^4,Gly^7]OT binding to hippocampal membranes (Fig. 2) revealed a single class of binding sites, thus, contrasting with the curvilinear Scatchard of [^{3}H]OT binding obtained by Audigier and Barberis (2). Binding to lactating mammary gland, estrogenized, and OVX myometrial membranes also appeared to take place at a single class of binding sites (Fig. 2). As indicated in Table 2 the mean K_d values for [^{3}H]-[Thr^4,Gly^7]OT binding to membranes from the hippocampus, uterus, and mammary gland were similar but the total binding capacities varied greatly with the tissue. In the ventral hippocampus the number of detected sites was 44 ± 8 fmol/mg protein. The myometrial membrane binding capacity of uteri from 17β-estradiol-treated animals (730 ± 264 fmol/mg protein) was about four times higher than that found for uteri from OVX animals (182 ± 90 fmol/mg protein). Because of the small number of binding sites no further experiments were carried out with the OVX uteri. Lactating mammary gland tissue also has a high number of binding sites (592 ± 214 fmol/mg protein).

Ligand specificity of [^{3}H]-[Thr^4,Gly^7]OT binding sites. To ascertain that [^{3}H]-[Thr^4,Gly^7]OT does label OT receptors in estrogenized myometrial membranes competition experiments with the unlabeled hormones and analogues listed in Table 1 were performed. Typical experiments are illustrated in Fig. 3*A*, and the results of the entire series of experiments are summarized in Table 3. A comparable maximal displacement of [^{3}H]-[Thr^4,Gly^7]OT binding was obtained with all the ligands tested, whereas displacement curves were parallel and their slopes (calculated as in Table 3) not significantly different from unity. Furthermore, the nonspecific component of binding was the same with a 250-fold excess OT or [Thr^4,Gly^7]OT. Together these observations indicate [^{3}H]-[Thr^4,Gly^7]OT labeling of one single population of OT receptors in the estrogenized uterus. Computer analysis of these binding data with LIGAND (17 curves) indicated that with a two-site model the fit was not better than with a one-site model.

Unlabeled [Thr^4,Gly^7]OT inhibited [^{3}H]-[Thr^4,Gly^7]-OT binding with a high affinity (K_i = 1.0 ± 0.4 nM), comparable to the value derived from concentration dependency experiments (K_d = 1.0 ± 0.6 nM). OT also had a high affinity for the presumed OT receptor (K_i = 1.0 ± 0.1 nM). A recently synthesized potent OT antagonist desGly(NH$_2$)^{9}d(CH$_2$)$_5$[Tyr(Me)^{2}Thr^4]OVT (Sawyer, Bankowski, and Manning, unpublished results) shown to have a very low antiantidiuretic and a low antivasopressor potency (Table 1) inhibited [^{3}H]-[Thr^4,Gly^7]OT binding with high affinity (K_i = 3.2 ± 0.2 nM). It was checked that, as expected, desGly(NH$_2$)^{9}d(CH$_2$)$_5$-[Tyr(Me)^{2}Thr^4]OVT binds to V_{1a} vasopressin receptors on rat liver membranes with low affinity (K_d = 520 ± 40 nM). Although initially no data were available on the affinity of [Thr^4,Gly^7]OT for V_2 receptors, the antagonist d(CH$_2$)$_5$[D-Phe^{2}Ile^4]AVP, with enhanced antiantidiuretic potency and reduced antioxytocic and antivasopressor potencies (20), was used as a selective ligand for V_2 vasopressin receptors. It inhibited [^{3}H]-[Thr^4,Gly^7]OT

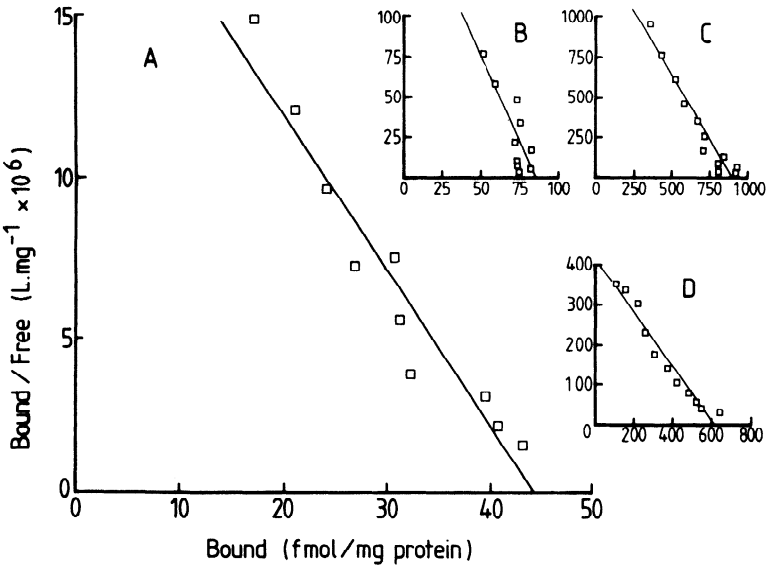

FIG. 2. Concentration dependency of [^{3}H]-[Thr^4,Gly^7]OT binding to ventral hippocampal synaptic plasma membranes (*A*) (1.0–30.0 nM), membranes of ovariectomized (*B*) (0.3–32.0 nM) and estrogenized uterus (*C*) (0.3–32.0 nM), and lactating mammary gland (*D*) (0.3–32.0 nM). Nonspecific binding was determined with a 250-fold excess of [Thr^4,-Gly^7]OT or OT. Each *curve* is mean of triplicate determinations of 1 experiment. Dissociation constants and maximal binding capacities (see Table 2) were estimated from calculated regression lines.

TABLE 2. *Binding characteristics of [^{3}H]-[Thr^4,Gly^7]OT to purified membrane preparations*

Tissue	K_d, nM	B_{max}, fmol/mg protein	n
Ventral hippocampus	2.0±0.9	44±8	6
Uterus, estrogenized	1.0±0.6	730±264	6
Uterus, OVX	1.5±2.1	182±90	3
Mammary gland, lactating	1.3±0.2	592±214	6

Values are means ± SD of 3–6 determinations and are derived from Scatchard evaluations of the experimental data base. Membranes are incubated with different concentrations of [^{3}H]-[Thr^{4}Gly^7]oxytocin (OT) with or without the presence of a 250-fold excess of OT. Analyses of binding, conducted with LIGAND, gave no indication of binding to more than one population of sites (P values for a one-site fit were $P <$ 0.001 for ventral hippocampal, $P = 1.000$ for estrogenized uterus, $P <$ 0.002 for uterus OVX, and $P = 0.298$ for lactating mammary gland membranes). Estrogenized uterus indicates treatment with 17β-estradiol benzoate; OVX, ovariectomy (see MATERIALS AND METHODS).

binding to myometrial membranes with an affinity (K_i = 14.6 ± 7.6 nM) ~15 times lower than those of OT or [Thr^4,Gly^7]OT. Altogether these results indicate that [^{3}H]-[Thr^4,Gly^7]OT does label OT receptors in myometrial membranes but not vasopressin receptors of the V_1 or V_2 subtypes.

AVP, which possesses a high affinity for hippocampal OT receptors (2) but only a limited capacity to stimulate uterus contraction (Table 2), appeared to have a comparable high affinity for [^{3}H]-[Thr^4,Gly^7]OT binding sites from myometrial membranes (K_i = 1.7 ± 0.4 nM). AVT, having intermediate capacities in stimulating uterus (Table 2), also displayed a high affinity (K_i = 0.6 ± 0.1 nM).

The unlabeled ligands used to characterize OT receptors from myometrial membranes were also used to compare the ligand selectivities of OT receptors from the different tissues tested (Fig. 3 and Table 3). Analysis of the binding data with LIGAND of two sets of 16 curves, obtained in ventral hippocampal and lactating mammary gland membranes, indicated an adequate fit to one category of binding sites.

Unlabeled [Thr^4,Gly^7]OT inhibited [^{3}H]-[Thr^4,Gly^7]-OT binding to ventral hippocampal and mammary gland membranes with affinity constants (K_i = 1.9 ± 0.5 and 0.8 ± 0.2 nM, respectively) that are close to the corresponding dissociation constants (K_d = 2.0 ± 0.9 and 1.3 ± 0.2 nM) derived from Scatchard analyses. AVP and AVT, having only limited milk ejection capacities in the rabbit mammary gland (Table 2), have high affinities for the [^{3}H]-[Thr^4,Gly^7]OT binding sites from the rat mammary gland (K_i = 2.0 ± 0.2 and 0.7 ± 0.4 nM) and ventral hippocampal membranes (K_i = 1.6 ± 0.1 and 1.9 ± 0.6 nM). The antagonist d(CH$_2$)$_5$[D-Phe^{2}Ile^4]AVP had a moderately low affinity of 21.1 ± 11.6 and 26.3 ± 12.1 nM, respectively. Conclusively, none of the used ligands revealed heterogeneity in the population of [^{3}H]-[Thr^4,Gly^7]OT binding sites in the ventral hippocampus, estrogenized uterus, and mammary gland.

Analysis of [^{3}H]AVP binding to estrogen-treated uterus (K_d = 0.8 ± 0.3 nM) and lactating mammary gland (K_d = 1.4 ± 0.4 nM) membrane preparations confirmed the high affinity of AVP for the binding sites on these membranes. Nonspecific binding at a concentration of 1 nM was 7 and 14%, respectively, of the total binding. The maximal binding capacity of [^{3}H]AVP binding sites, 541 ± 65 and 463 ± 227 fmol/mg protein, respectively, was not significantly different from that of [^{3}H]-[Thr^4,Gly^7]OT binding sites. Specific [^{3}H]AVP binding could be completely displaced by [Thr^4,Gly^7]OT with an affinity of 0.6 ± 0.2 nM in the estrogenized uterus (Fig. 4) and 2.8 ± 1.9 nM in the lactating mammary gland. Simultaneous analysis of the [^{3}H]AVP binding experiments with LIGAND showed an adequate fit to a one-site model in both cases, $P = 0.840$ (uterus) and $P = 0.889$ (mammary gland) for a two-site model.

The unexpected high affinity of AVP for [^{3}H]-[Thr^4,Gly^7]OT binding sites in uterus and mammary gland urged us to examine a possible involvement of high Mg^{2+} concentrations in affinity regulation of OT receptors. The potency of OT and AVP to displace [^{3}H]-[Thr^4,Gly^7]OT (1.8 nM) binding to estrogenized myometrial membranes was determined in competition experi-

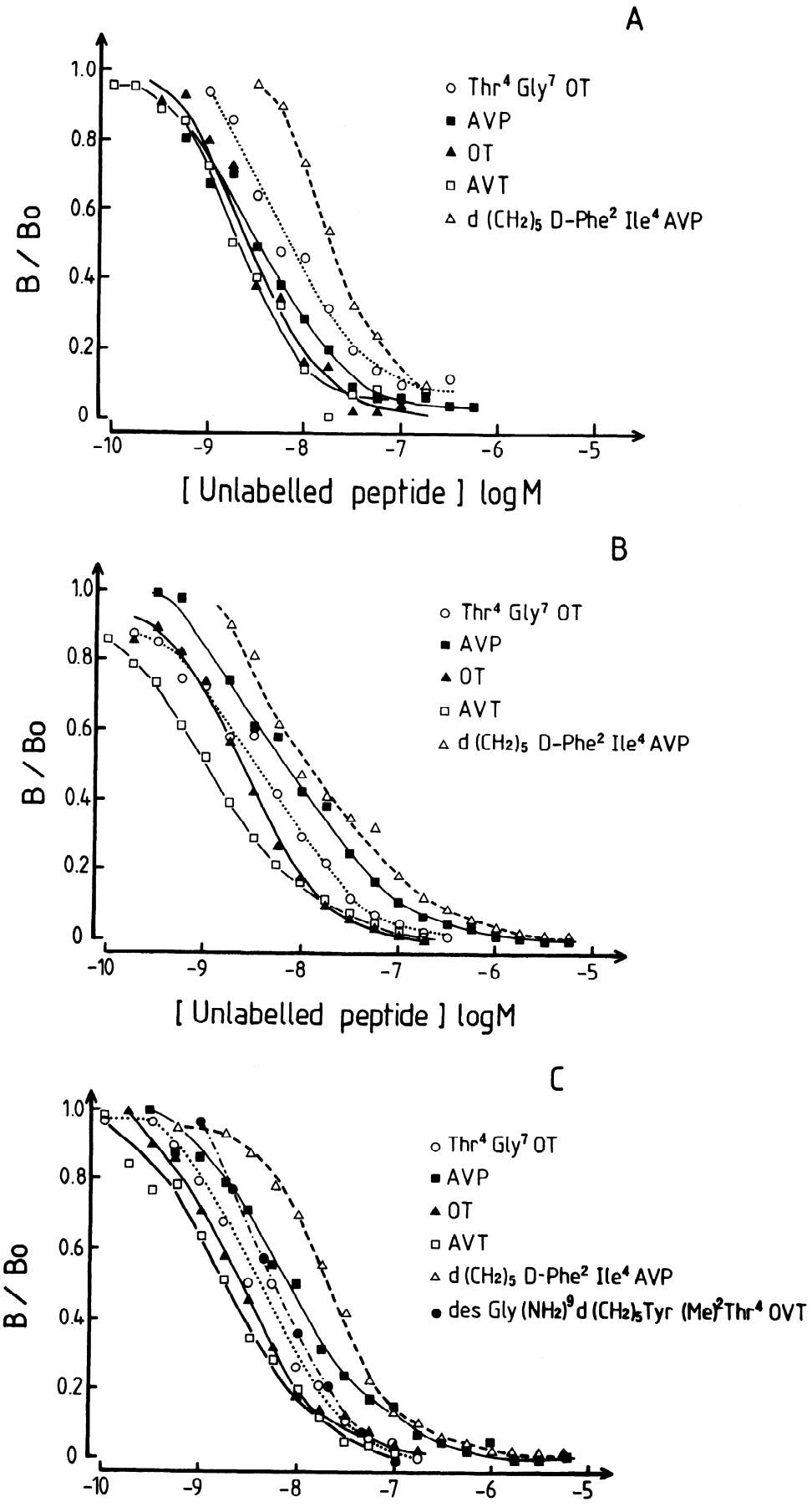

FIG. 3. Dose-dependent displacement of [^{3}H]-[Thr^4,Gly^7]OT binding to hippocampal (*A*), lactating mammary gland (*B*), and estrogenized uterine (*C*) membrane preparations by unlabeled peptides mentioned in Table 1. Membranes were incubated in the presence of a constant amount of [^{3}H]-[Thr^4,Gly^7]-OT (1.7–2.0 nM) and various amounts of unlabeled peptides. Each *curve* is mean of triplicate determinations of 1 experiment.

TABLE 3. *Affinities of OT, AVP, and their analogues for [^{3}H]-[Thr^4,Gly^7]OT binding sites*

Ligand	Ventral Hippocampus	Uterus Estrogen	Lactating Mammary Gland
AVP	1.6±0.1	1.7±0.4	2.0±0.2
OT	1.9±1.0	1.0±0.1	1.0±0.2
AVT	1.9±0.6	0.6±0.1	0.7±0.4
[Thr^4,Gly^7]OT	1.9±0.5	1.0±0.4	0.8±0.2
d(CH$_2$)$_5$[D-Phe^{2}Ile^4]AVP	26.3±12.1	14.6±7.6	21.1±11.6
desGly(NH$_2$)^{9}d(CH$_2$)$_5$[Tyr-(Me)^{2}Thr^4]OVT		3.2±0.2	

Inhibition constants obtained from dose-dependent competition of [^{3}H]-[Thr^4,Gly^7]oxytocin (OT) binding to indicated membrane preparations. Membranes were incubated in presence of a constant amount of [^{3}H]-[Thr^4,Gly^7]OT (1.7–2.0 nM) and various amounts of unlabeled peptides. Slope index and K_i values were calculated by fitting the experimental data to expected linear relationship $\log[(B_0/B) - 1][([^3H]/K_d{}^3H) + 1] = \log[I] - \log(K_i)$, in which B_0 represents specific binding in absence and B the specific binding in presence of unlabeled competitor. [I] is the concentration of [^{3}H]-[Thr^4,Gly^7]OT. $K_d{}^3$H is the dissociation constant at equilibrium of [^{3}H]-[Thr^4,Gly^7]OT in incubation medium. Families of homologues and heterologues displacement experiments were analyzed with LIGAND. Fitting the data to a two-site model was not significantly better than to a one-site model ($P = 1.000$ for ventral hippocampus, $P = 0.789$ for uterus estradiol, and $P = 1.000$ for mammary gland membranes). Each K_i represents the mean ± SD of two independent experiments in triplicate. Uterus estrogen indicates 17β-estradiol treatment (see MATERIALS AND METHODS).

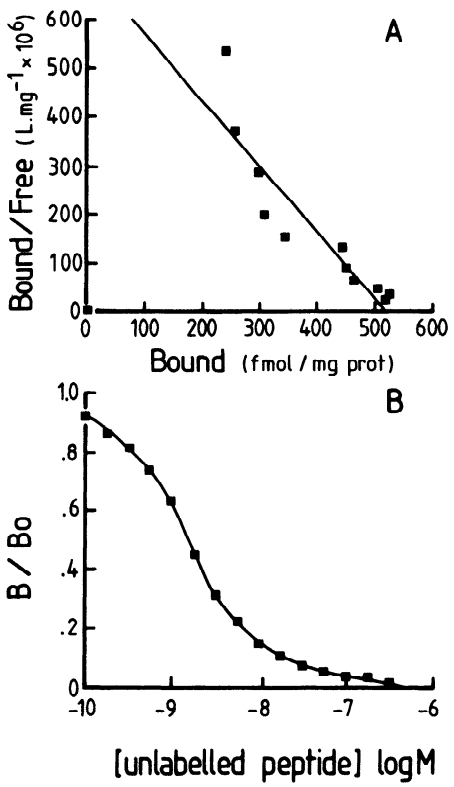

FIG. 4. [^{3}H]AVP binding to estrogenized membrane preparations. *A*: membranes were incubated with 0.4–40 nM [^{3}H]AVP. $K_d = 0.8 \pm 0.3$ nM, $B_{max} = 540 \pm 45$ fmol/mg protein. *B*: dose-dependent displacement of [^{3}H]AVP binding (2.0 nM) by [Thr^4,Gly^7]OT. $K_i = 0.6 \pm 0.2$ nM. Nonspecific binding in *A* and *B* was determined with a 250-fold excess AVP. Each *curve* is mean of triplicate determinations of 1 experiment.

ments at Mg^{2+} concentrations of 10 mM, used in this study, and 1 mM, a concentration close to that often used in biological assays. Total [^{3}H]-[Thr^4,Gly^7]OT binding at 1 mM Mg^{2+} was ~64% of the value at 10 mM Mg^{2+}, whereas nonspecific binding remained unchanged. The ratio of half-maximal displacement values at 10 mM and 1 mM Mg^{2+} is 0.64 for AVP and 0.60 for OT.

DISCUSSION

Previous studies on OT receptors have used [^{3}H]OT as labeled ligand. Although [^{3}H]OT discriminates between OT and AVP receptors, its affinity for AVP receptors is high enough to generate complex binding curves in all situations where both OT and AVP receptors are present in the same preparation. This was shown to be the case for rat hippocampal synaptosomal membranes (2) and human myometrial membranes (15). We show here that [^{3}H]-[Thr^4,Gly^7]OT is a much more selective radioligand for OT receptors. The conclusion that [^{3}H]-[Thr^4,Gly^7]OT does label OT receptors selectively was based on the following observations: *1*) [Thr^4,Gly^7]OT was shown to be a potent oxytocic agonist in uterus and mammary gland preparations (18); *2*) [^{3}H]-[Thr^4,Gly^7]-OT binds with high affinity to a single population of binding sites on membranes prepared from OT receptor-rich tissues (uterus and mammary gland); *3*) [^{3}H]-[Thr^4,Gly^7]OT binding is inhibited to the same maximal extent by unlabeled OT and [Thr^4,Gly^7]OT; *4*) [^{3}H]-[Thr^4,Gly^7]OT binding sites exhibit a high affinity for OT in all tissues tested. In the estrogenized uterus, [^{3}H]-[Thr^4,Gly^7]OT binding was displaced with a high affinity by a highly specific oxytocic antagonist, desGly(NH$_2$)^{9}d(CH$_2$)$_5$[Tyr(Me)^{2}Thr^4]OVT; *5*) [Thr^4,Gly^7]OT binds with a very low affinity (K_i = 3700 nM) to V_1-type vasopressin receptors on rat superior cervical ganglion (17) and rat liver membranes (K_i > 8,000 nM, Elands and Barberis, unpublished observations), V_2-type vasopressin receptors on LLC-PK$_1$ cells (K_i > 10,000 nM, Cantau et al., personal communication), and to vasopressin receptors on pig epididymis [K_i > 10,000 nM, Maggi et al. (19)]; *6*) on ventral hippocampal membranes that contain both OT and AVP receptors, Scatchard analysis of [^{3}H]-[Thr^4,Gly^7]OT binding data revealed binding with high affinity ($K_d = 2.0 \pm 0.5$ nM) to one class of receptors.

[^{3}H]AVP binding sites in estrogenized uterus and lactating mammary gland membranes were probably identical to [^{3}H]-[Thr^4,Gly^7]OT binding sites on these tissues. AVP was shown to have a high affinity for [^{3}H]-[Thr^4,Gly^7]OT binding sites, [Thr^4,Gly^7]OT completely displaced the [^{3}H]AVP binding with a high affinity, binding appeared to take place to a single population of binding sites, and the number of binding sites corresponds to the number of [^{3}H]-[Thr^4,Gly^7]OT binding sites. In the rat hippocampus, where AVP and OT receptors are present, [^{3}H]AVP bound to both binding sites with an equally high affinity. However, the displacement of [^{3}H]AVP binding by OH[Thr^4,Gly^7]OT, a related [Thr^4,Gly^7]OT analogue, was biphasic, clearly indicating [^{3}H]AVP binding to two different receptor classes (2). This was clearly not the case in the estrogenized uterus,

suggesting that [^{3}H]AVP only binds to the OT receptors.

The data derived from the present characterization of OT receptors from rat uteri and lactating rat mammary glands with [^{3}H]-[Thr^4,Gly^7]OT as labeled ligand compare very nicely with previously published data obtained with [^{3}H]OT. Soloff and Swartz (29, 30) reported maximal binding capacities of 180 fmol/mg protein in intact estrogen-treated rat uterus and 280 fmol/mg protein in lactating mammary gland. Partly, the difference between our results (730 ± 264 fmol/mg protein for estrogen-treated rat uteri and 592 ± 214 fmol/mg protein in rat-lactating mammary gland) can be explained by a difference in membrane purity. Soloff and Swartz used 20,000 *g* fractions instead of purified membranes. Furthermore, in their studies, rats were treated for 2 days with estrogen instead of 7 in the present study. The observed increase in the OT binding capacity of myometrial membranes after estradiol treatment is comparable to that reported by Fuchs et al. (14). The maximal binding capacity in the ventral hippocampus was estimated at 44 ± 8 fmol/mg protein, whereas 16.9 fmol/mg protein was calculated for the total hippocampus (2), a difference that can be accounted for by the fact that OT binding sites are mainly found in the ventral part of the hippocampus (8, 12).

The main contribution of the present study is to show that OT receptors from the ventral hippocampus exhibit properties that are very similar to those of OT receptors from rat uterus and lactating mammary gland with regard to their affinity for [^{3}H]-[Thr^4,Gly^7]OT and their specificities for the tested ligands. The hypothesis that hippocampal OT receptors might be different from peripheral types was based on the observation that hippocampal receptors discriminate very poorly between OT and AVP (2). We show here that the same conclusion applies to OT receptors from the rat uterus and mammary gland. Data on the affinity of OT receptors for vasopressin are in fact very scarce. Soloff and co-workers only measured affinities of lysine-vasopressin (LVP). Estimations for LVP are 17% of the OT affinity in the rat uterus (30) and 16–22% in the rat-lactating mammary gland (27, 29). Affinities of LVP and AVP for OT receptors are, however, not equal. Guillon et al. (15) reported a lower affinity for LVP (K_i = 15.3 nM) than for AVP (K_i = 1.2 nM) in human myometrium. By use of a crude rat uterus membrane preparation Antoni (1) observed for OH[Thr^4,Gly^7]OT an affinity of ~10% and for OT of ~20% of that of AVP, whereas our results indicate a higher affinity of AVP for uterine and mammary gland OT receptors. In line with our results OT binding sites in the male genital tract of pigs were shown to have a high affinity for [Thr^4,Gly^7]OT, AVP, and AVT (19).

The observation that AVP binds with high affinity to OT receptors from the rat uterus and mammary gland is surprising in view of the low biological potency of AVP compared with OT. AVP only has 5% of the OT capacity to elicit uterus contraction (at 0.5 mM Mg^{2+}) in the rat and 15% to induce a milk-ejection reflex in the rabbit (51 and 46% for AVT, respectively). The efficiency of Postlobin-V and of Pitressin (early vasopressin preparations) in contracting, respectively, guinea pig uterus in vitro (11) and cow uterus in vivo (10) was, however, reported to equal that of, respectively, Postlobin-O and Pitocin (early oxytocin preparations), when Mg^{2+} concentration in the bathing solution was raised up to 10 mM (the concentration used in our study). It is also clear from studies by Pearlmutter and Soloff (23) that Mg^{2+} and other divalent cations do play a role in regulating affinity and capacity of OT receptors. Recently Soloff and Grzonka (28) demonstrated a selective enhancement by Mn^{2+} of the relative affinities for OT receptors in rat myometrial and mammary gland membranes of a series of OT analogues with an inversely decreasing affinity at low Mn^{2+} concentrations. These observations raise the possibility that the observed high affinity of AVP for OT receptors was related to the Mg^{2+} concentration used in our binding assay. Although we confirm that high Mg^{2+} concentrations did enhance the [^{3}H]-[Thr^4,Gly^7]OT binding (at 10 mM Mg^{2+} specific binding was 1.5 times higher than at 1 mM Mg^{2+}), the relative affinities of OT and AVP were similar at a Mg^{2+} concentration of 1 and 10 mM. Therefore, we tentatively conclude that the absence of discrimination between OT and AVP is an intrinsic property of these OT receptors. The apparent discrepancy between endocrinological activities of OT and AVP in contracting uterus and inducing a milk ejection reflex and the here presented affinities for the OT receptor might eventually be explained by differences in intrinsic activity of OT and AVP for OT receptors in a situation where the spare receptor phenomenon is operative. This suggests that AVP might be a partial agonist at physiological Mg^{2+} levels. High Mg^{2+} concentrations can be thought of as enhancing intrinsic activity of AVP for OT receptors in uterus and mammary gland as could be the case for the elevated potency of Postlobin-V and Pitressin in contracting uterus in the presence of 10 mM Mg^{2+}.

In summary, the present study shows that OT receptors from synaptic plasma membranes from the rat hippocampus and OT receptors from the uterus and mammary gland exhibit striking similarities with respect to their relative affinities for the natural hormones AVP, OT, and vasotocin and for the highly selective OT agonist [Thr^4,Gly^7]OT.

However, studies with a much larger series of structural analogues are needed before one can conclude whether central and peripheral OT receptors are identical or not. Neither can it be judged whether OT receptors in the lactating mammary gland and estrogenized uterus are similar or not. Finally, not all OT receptors are identical; rat adipocytes for example are reported to have OT receptors with ~100 times lower affinity for AVP (4).

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Present address of J. Elands: Rudolf Magnus Institute for Pharmacology, University of Utrecht, Vondellaan 6, 3521 GD, Utrecht, The Netherlands.

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