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Pharmacological characterization of two specific binding sites for neurohypophyseal hormones in hippocampal synaptic plasma membranes of the rat

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Synaptic plasma membranes containing binding sites for tritiated oxytocin and arginine vasopressin were isolated from rat hippocampus. The binding parameters for oxytocin and vasopressin sites were determined and statistically analysed. The fitted curve for oxytocin binding was compatible with a model where the ligand interacts with two classes of receptors with different capacities and affinities. The sites with low binding capacity had an apparent dissociation constant at equilibrium of 1.8 nM and a maximal binding capacity of 17 fmol/mg protein. By contrast, the Scatchard plot failed to reveal a marked heterogeneity in the population of sites labelled with [^{3}H]vasopressin with an affinity of 1.5 nM and a maximal binding capacity of 39 fmol/mg protein. The specificity of these binding sites, tested in competition experiments, revealed that these neurohypophyseal hormones labelled two distinct populations of sites. One population with a high affinity for vasopressin, oxytocin and vasotocin, the other population with a high affinity for vasopressin and vasotocin and a low affinity for oxytocin. Adenylate cyclase activity was not affected by arginine-vasopressin or oxytocin. These receptors are compared with previously characterized peripheral receptors.

Key words: oxytocin/vasopressin/binding sites/hippocampus

Introduction

Vasopressin and oxytocin have long been recognized as the neurohypophyseal hormones carried by a vascular route to affect peripheral target tissues. More recently, immunocytochemical techniques have provided evidence that a direct transport of vasopressin and oxytocin within the brain occurs *via* long axonal projections from the hypothalamus to several extrahypothalamic regions (for reviews, see Kozlowski *et al.*, 1983; Buijs, 1983). These studies, and other observations that neurohypophyseal peptides have several behavioural effects including an influence on the retention of conditioned avoidance tasks in rats (De Wied, 1980), have led to the conclusion that these hormones may also have neural functions. Thus arginine-vasopressin binding sites have been shown in brain tissue using either autoradiographic techniques (Baskin *et al.*, 1983; Van Leeuwen and Wolters, 1983; Yamamura *et al.*, 1983; Biegon *et al.*, 1984; De Kloet *et al.*, 1985) or radioligand binding techniques (Pearlmutter *et al.*, 1983; Dorsa *et al.*, 1983). In a previous study, we characterized specific lysine vasopressin binding sites to rat hippocampal membranes, suggesting that vasopressin receptors are present in the hippocampus (Barberis, 1983). The particularly interesting electrophysiological study of Muhlethaler *et al.* (1983) suggested that a class of hippocampal neurons is endowed with receptors for oxytocin. This means that both vasopressin and oxytocin receptors may be present in the hippocampus. Here we report the characterization of two specific binding sites for neurohypophyseal hormones in the rat hippocampus and compare them with the previously characterized binding sites for vasopressin (Jard, 1983) and oxytocin (Soloff *et al.*, 1977).

Results

Rat hippocampal synaptic plasma membranes were found to bind [^{3}H]oxytocin specifically. In the presence of 3.2 nM of labelled ligand, the specific component of binding, increased as a func-

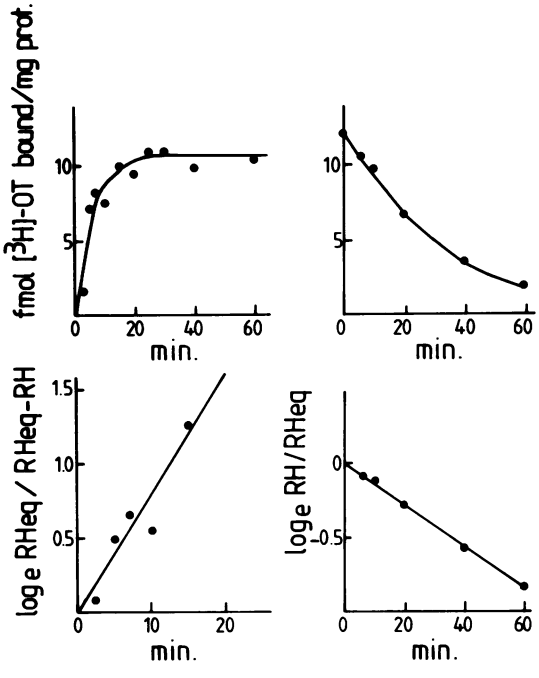

Fig. 1. Time-course of the association of [^{3}H]oxytocin with its high-affinity binding sites; reversibility of binding. Synaptic plasma membranes (260 μg protein) were incubated in a final volume of 200 μl in the presence of [^{3}H]oxytocin (3.2 nM). Oxytocin binding was measured as a function of time as indicated under Materials and methods. To test binding reversibility, the enzyme was first incubated for 20 min at 30°C in the presence of [^{3}H]oxytocin (3.0 nM). Unlabelled oxytocin (1 μM final concentration) was then added and total residual binding measured as a function of time after addition of unlabelled hormone. The K_{+1} and K_{-1} (association and dissociation constants) were determined from the semilogarithmic plots shown in the lower part of the figure assuming: (i) that the binding process corresponds to the equilibrium

$$H + R \underset{K_{+1}}{\overset{K_{-1}}{\rightleftharpoons}} RH$$

in which H, R, and RH are, respectively, free hormone, free receptor and the hormone receptor complex; (ii) that the free hormone concentration is constant. The association and dissociation time-courses are described by equations 1 and 2, respectively:

(1) $\log_e[RH\text{eq}]/([RH\text{eq}] - [RH]) = (K_{+1}[H] + K_{-1})t$

(2) $\log_e[RH]/[RH\text{eq}] = K_{-1}t$ with $[RH\text{eq}]$ = concentration of hormone receptor complex at equilibrium. Values on the graph were obtained in three independent experiments.

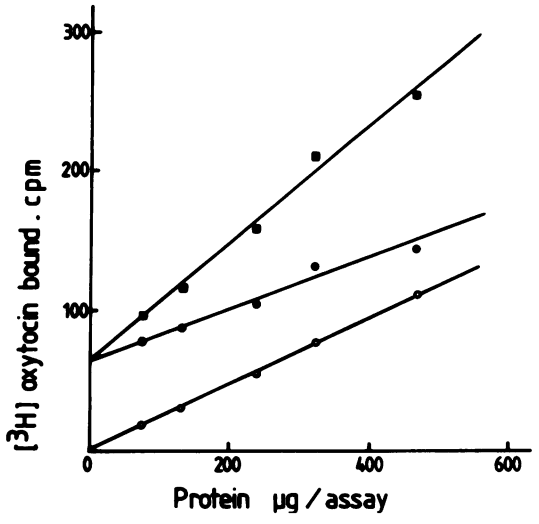

Fig. 2. Correlation between [^{3}H]oxytocin binding and membrane concentration. Purified synaptic plasma membranes were incubated as indicated in the Materials and methods in the presence of 2.4 nM [^{3}H]oxytocin. Values on the graph are means of three determinations. S.E.M. are always <8.1%. Values of the correlations calculated for the regression lines were: $r = 0.995$; $r = 0.978$ and $r = 0.999$ for the total binding, non-specific binding and specific binding, respectively. (■) Total binding; (●) non-specific binding; (○) specific binding.

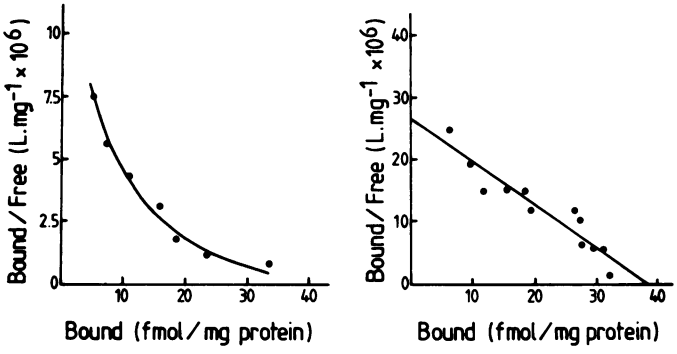

Fig. 3. Dose-dependence for specific [^{3}H]oxytocin **(left)** and [^{3}H]arginine-vasopressin **(right)** to purified hippocampal synaptic plasma membranes. The graphs show the Scatchard plot of the dose-dependent binding curves. Values on the graphs are means of four independent experiments. Values of the dissociation constants and maximal binding capacities are estimated at 1.78 nM and 16.9 fmol/mg protein for [^{3}H]oxytocin high-affinity binding sites and 30 nM and 25 fmol/mg protein for [^{3}H]oxytocin low-affinity binding sites, respectively. Non-linear weighted ($1/\sigma^2$) least-squares regression analysis of untransformed data was conducted on a Tektronix 4052 computer. Testing for statistically significant resolution of two receptor subtype components is obtained by using a partial F-test (see Munson and Rodbard, 1980). Saturation isotherm was best-fitted ($p < 0.05$) with a two-site model. For [^{3}H]arginine vasopressin, the dissociation constants and maximal binding capacities estimated from the calculated regression lines are 1.46 nM and 38.8 fmol/mg protein, respectively.

tion of time, reached an equilibrium state within a 20 min incubation period and was stable thereafter up to 60 min. The binding half-time was 5 min (Figure 1). All further experiments were performed at 30°C and the duration of membrane incubation in the presence of [^{3}H]oxytocin was 20 min. Specifically bound [^{3}H]oxytocin could be released in a time-dependent manner by adding an excess of unlabelled oxytocin (1.5 µM). After 60 min incubation in the presence of unlabelled oxytocin <15% of radioactivity bound at zero-time remained attached to the membranes (Figure 1). Both the association and dissociation time-courses were mono-exponential processes (Figure 1). The rate constant for the association reaction K_{+1} was 0.050 x 10^9/mol/min and the rate constant for the dissociation reaction K_{-1} was 0.028/min.

The specific or non-specific component of [^{3}H]oxytocin binding at equilibrium increased linearly with the membrane concentration in the incubation medium up to 470 µg protein/assay (Figure 2). As Figure 2 shows a large fraction of the apparent non-specific component of binding could be accounted for by radioactivity retained on the filter. At a concentration of 0.6 nM [^{3}H]oxytocin and a membrane concentration of 200−300 µg protein/assay, the non-specific component of [^{3}H]oxytocin binding (non-specific binding to membranes plus non-specific binding to filter) represented 61.8 ± 2.7% of total binding (mean ± SD of eight determinations). Finally h.p.l.c. analysis revealed that under the experimental conditions used for all further experiments (see Materials and methods), [^{3}H]oxytocin was not inactivated during the course of incubation in the presence of membranes.

Figure 3 shows the Scatchard plot of the dose-dependent specific binding of [^{3}H]oxytocin at equilibrium. Clearly this plot was curvilinear. Computerised fitting of the data with a multisite model indicated that the best fit was obtained by assuming that oxytocin binds to two categories of sites of high and low affinity. Calculated values for dissociation constants (K_d) and maximal binding capacities (B_{max}) were 1.8 nM and 16.9 fmol/mg protein and 30 nM and 25 fmol/mg protein for high and low affinity sites, respectively.

The ligand specificity of oxytocin binding sites was evaluated from competition experiments similar to those illustrated by Figure 4, using the series of oxytocin and vasopressin analogues listed in Table I. Since [^{3}H]oxytocin was used at a concentration of 3 nM, one can estimate that, in the absence of unlabelled ligand, >80% of bound radioactivity was attached to the high affinity sites. The results derived from competition experiments are therefore representative of the ligand specificity of the high affinity oxytocin binding sites. Data shown in Figure 4 indicated the following. (i) The K_i value for oxytocin binding calculated from competition experiments (2.1 nM) was very close to the K_d (1.8 nM) deduced from a direct determination of [^{3}H]oxytocin binding. This observation indicates that unlabelled- and [^{3}H]oxytocin have very comparable properties. (ii) The detected high affinity oxytocin binding sites discriminated very efficiently between analogues exhibiting enhanced oxytocic or vasopressor selectivity on peripheral organs. Thus the selective oxytocic agonist (OH-Thr^4, Gly^7)-OT inhibited [^{3}H]oxytocin binding with a high potency (K_i = 2.3 nM) and the selective vasopressor agonist Phe^{2}Orn^{8}VT had a low affinity (K_i = 58 nM). (iii) Surprisingly the high affinity oxytocin binding sites discriminated very poorly between oxytocin and vasopressin (respective K_i values of 2.1 and 3.7 nM). Arginine vasotocin had a slightly higher affinity than oxytocin. It was difficult to explore the ligand specificity of the low affinity oxytocin sites. This would have needed the use of high [^{3}H]oxytocin concentration with the expected consequence of having a very unfavourable specific/non-specific binding ratio.

Although the experiments described above indicated that the binding sites labelled with [^{3}H]oxytocin at low concentrations were different from those labelled by [^{3}H]vasopressin (Barberis, 1983), the observation that the hippocampal binding sites discriminated very poorly between oxytocin and vasopressin raised the possibility that [^{3}H]vasopressin might also label the detected oxytocin binding sites. This encouraged us to perform additional experiments with [^{3}H]vasopressin. Dose-dependent specific bind-

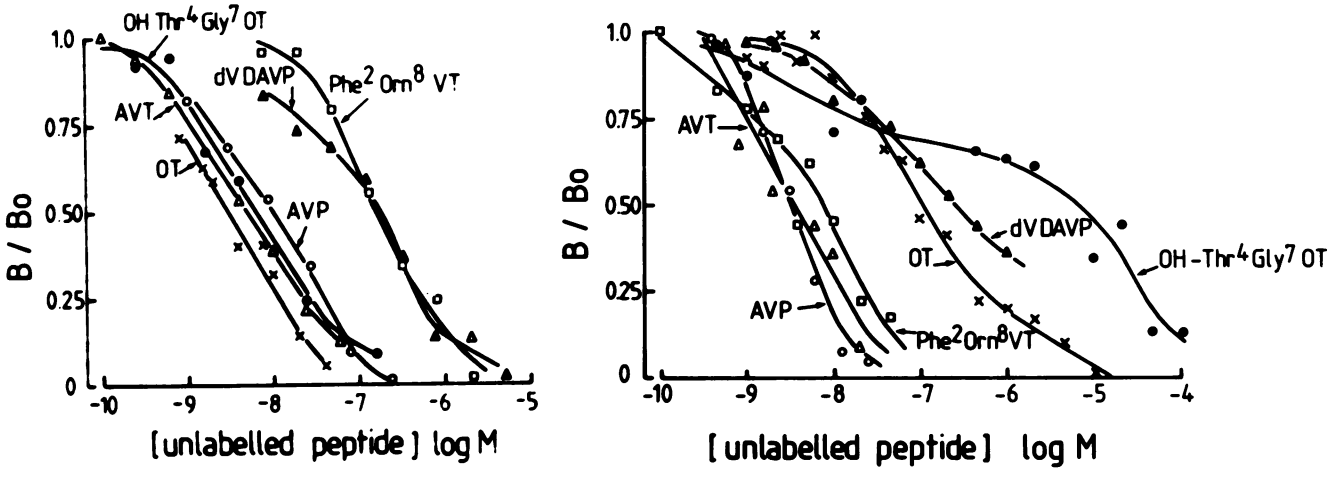

Fig. 4. Dose-dependent inhibition of [^{3}H]oxytocin **(left)** and [^{3}H]vasopressin **(right)** binding to hippocampal synaptic plasma membranes by unlabelled peptides. Membranes were incubated in the presence of a constant amount of [^{3}H]oxytocin (3 nM) or [^{3}H]vasopressin (1 nM) and increasing amounts of unlabelled peptides. Values of specific binding measured in the presence of unlabelled peptides (B) were expressed as a fraction of the specific binding measured in the absence of competitor (Bo). Each point is the means of triplicate determinations. The apparent dissociation constants (K_i) for the non-labelled peptides were calculated by using the following relation: $K_i = I_{50} \times K_d{*}H/(K_d{*}H + [{*}H])$ in which I_{50} is the concentration of unlabelled peptide leading to half-maximal inhibition of labelled hormone specific binding. $K_d{*}H$ is the dissociation constant at equilibrium for tritiated hormone binding and $[{*}H]$ the concentration of tritiated hormone in the incubation medium. The slope index of the displacement curves was deduced by fitting the experimental data to the expected linear relationship: $[(Bo/B) - 1]\ [{*}H/K_d{*}H) + 1] = \log(I) - \log K_i$, in which (I) is the concentration of unlabelled peptide.

Table I. Endocrine activities of oxytocin, arginine vasopressin and analogues

	Relative biological activities[a]			
	Oxytocic[b]	Antidiuretic[c]	Vasopressor[c]	Hippocampal[b]
– Oxytocin (OT)	100	1.2	1.1	100.0
– Arginine vasotocin (AVT)	51	91.0	62.0	81.6
– [1-(L-2-hydroxy-3-mercapto-propionic acid)-threonine^4, glycine^7]-oxytocin (OH-Thr^{4}Gly^{7}OT)	206	0.001	<0.001	96.8
– arginine vasopressin (AVP)	5.3	100.0	100.0	52.5
– (phenylalanine^2, ornithine^8)-vasotocin (Phe^{2}Orn^{8}VT)	1.0	0.49	33.0	23.4
– 1-Deamino (valine^4, D-arginine^8)-vasopressin (dVDAVP)	0.4	380.0	Antagonist	6.9
– [1-(β-mercaptopropionic acid), 2-O-methyltyrosine, valine^4, D-arginine^8]-vasopressin [dTyr(Me)VDAVP]	Antagonist	619.0	Antagonist $pA_2=7.01$	Antagonist
– [1-(β-mercapto-β-β-cyclopentamethylene propionic acid), 2-O-ethyltyrosine, valine^4]-vasopressin [d$(CH_2)_5$Tyr(Et)VAVP]	Antagonist	Antagonist $pA_2=7.57\pm0.06$	Antagonist $pA_2=8.16\pm0.09$	

pA_2 is the negative log of A_2 which is the concentration of antagonists leading the response to 2 x units of oxytocin to equal the response to 1 x unit administered before the antagonist.
[a]Taken from Sawyer *et al.* (1981) and from Muhlethaler *et al.* (1983).
[b]Percent of oxytocin taken as a standard.
[c]Percent of arginine vasopressin taken as a standard.

ing at equilibrium was determined. The Scatchard plot failed to reveal (as already found) a marked heterogeneity in the population of sites labelled with [^{3}H] vasopressin with an affinity of 1.5 nM and a maximal binding capacity of 39 fmol/mg protein. It was clear, however, that the total number of sites labelled with [^{3}H]vasopressin was much higher than the maximal binding capacity of the 'so-called' high affinity 'oxytocin binding sites'. This observation is compatible with the hypothesis that [^{3}H]vasopressin could label both a population of sites exhibiting a high affinity for both oxytocin and vasopressin and a population of sites with a high (and similar) affinity for vasopressin and a much lower affinity for oxytocin. If this assumption is valid, we might expect that competition experiments with unlabelled analogues discriminating between the two populations of sites labelled with [^{3}H]vasopressin (among these is oxytocin, but not vasopressin) would lead to complex (shallow) displacement curves. Experiments shown in Figure 4 indicate that this was indeed the case.

The slope index of the displacement curve using unlabelled vasopressin was close to 1. The K_i value was 1.7 nM. With oxytocin the displacement curve was shallow (it covered a four-order of magnitude concentration range). The same situation was observed with OH-Thr^{4}Gly^{7}OT and to a lesser extent with Phe^{2}Orn^{8}VT and dVDAVP. With AVT and the antagonist d$(CH_2)_5$Tyr(Et)VAVP the displacement curve had a slope index close to 1. The main results are summarized in Table II. It is clear from Table II that tritiated neurohypophyseal hormones, oxytocin and vasopressin, very probably label two distinct populations of binding sites in the hippocampus. The results from the adenylate cyclase experiments are summarized in Table III. They show that, under experimental conditions where there was a 30% activation of the enzyme by vasoactive intestinal peptide, no

Table II. Pharmacological characterization of the binding sites of neurohypophyseal hormones

Site 1	Site 2
Labelled with high affinity with both [^{3}H]oxytocin and [^{3}H]vasopressin	Labelled with high affinity with [^{3}H]vasopressin and with low affinity with [^{3}H]oxytocin
High affinity for AVT	High affinity for AVT
High affinity for OH-Thr^{4}Gly^{7}OT	Low affinity for OH-Thr^{4}Gly^{7}OT
Low affinity for Phe^{2}Orn^{8}VT	High affinity for Phe^{2}Orn^{8}VT
dTyr(Me)VDAVP high affinity	Low affinity
d(CH$_2$)$_5$Tyr(Et)VAVP low affinity	High affinity

Table III. Adenylate cyclase activity in synaptic plasma membranes

Additions	Cyclic AMP formed (pmol/min/mg protein)
None	133 ± 8
VIP 1 μM	173 ± 4[a]
AVP 100 nM	134 ± 2
1 μM	133 ± 5
10 μM	134 ± 3
OT 100 nM	136 ± 2
1 μM	134 ± 5
10 μM	134 ± 1

Values in the table are means ± S.D. of 3–4 determinations. Values determined in the presence of vasoactive intestinal polypeptide (VIP), AVP or OT were compared with the corresponding basal values using one-tailed Student's t-test.
[a] $p < 0.01$.

change in activity could be detected in the presence of either oxytocin or vasopressin.

Discussion

Although many central actions have already been reported for oxytocin both *in vivo* (Morris *et al.*, 1980; Freund-Mercier and Richard, 1981; Joels and Urban, 1982) and *in vitro* (Muhlethaler *et al.*, 1983), the characterization of receptors located in the central nervous system has not been reported for oxytocin. Evidence for specific [^{3}H]oxytocin binding sites in central amygdala, ventral subiculum and olfactory nucleus have been recently provided by autoradiography techniques (Brinton *et al.*, 1984; De Kloet *et al.*, 1985). We show here that specific oxytocin binding sites can be detected on rat hippocampal synaptosomal membranes. Scatchard analysis of the dose-dependent binding curve at equilibrium revealed complex binding kinetics. Experimental data could be fitted with a model involving a reversible interaction of oxytocin with two independent populations of sites. Obviously other possibilities cannot be excluded on the basis of this observation. However, the comparison of binding data obtained with [^{3}H]oxytocin and [^{3}H]vasopressin makes it very likely that the complex kinetics of oxytocin binding reflects the existence of a cross-reactivity of oxytocin and vasopressin with two populations of sites,which, for the sake of simplicity, will be designated as oxytocin and vasopressin receptors. An unexpected observation derived from the present study is that oxytocin receptors (high-affinity oxytocin binding sites) do not discriminate efficiently between oxytocin and vasopressin. Furthermore, the affinity of vasopressin for oxytocin sites (K_i = 3.7 nM) is close to the affinity of vasopressin for vasopressin receptors (K_d = 1.5 nM). One could therefore expect that: (i) [^{3}H]vasopressin will label both vasopressin and oxytocin receptors; (ii) due to the small difference in the K_d values for binding of vasopressin to these receptors, analysis of the dose-dependency of vasopressin binding can hardly reveal this heterogeneity in the population of vasopressin sites.

In a previous study (Barberis, 1983) it was concluded on the basis of such an analysis that vasopressin ([^{3}H]lysine-vasopressin) interacted with a single population of sites on rat hippocampal synaptosomal membranes. We confirm in the present study that the [^{3}H]vasopressin binding data generated an almost linear Scatchard plot. However, using [^{3}H]vasopressin as the labelled ligand it is possible to find experimental conditions in which bound radioactivity will predominantly represent vasopressin molecules bound to vasopressin receptors, little (<20%) being attached to oxytocin receptors. In these conditions competition experiments with unlabelled peptides made it possible to estimate the affinity of these peptides for vasopressin receptors. Experiments with unlabelled oxytocin revealed that vasopressin receptors discriminate between oxytocin and vasopressin much more efficiently than do oxytocin receptors. Moreover, the displacement curve obtained with OH-Thr^{4}Gly^{7}OT (Figure 4) suggests that [^{3}H]vasopressin labelled two populations of sites with similar affinities for vasopressin. One of these populations might be the 'so-called' high-affinity oxytocin binding sites for which vasopressin has an affinity of 3.7 nM (B_{max} = 16.9 fmol/mg protein). The second population might be the high-affinity vasopressin binding sites for which vasopressin has an affinity of 1.5 nM and a maximal binding capacity of 22 fmol/mg protein. Likewise [^{3}H]oxytocin will label vasopressin and oxytocin receptors. In this case the difference in the affinity is high enough to allow a detection of the heterogeneity in oxytocin-binding sites from a direct analysis of dose-dependent [^{3}H]oxytocin binding.

The results shown in Figure 1 deserve special comment. Indeed, if oxytocin binds to two populations of sites, it is surprising to observe that the time courses of both hormonal bindings and reversal were mono-exponential processes. In fact at the [^{3}H]oxytocin concentration used in these experiments (3.2 nM) one can estimate that the bulk of bound radioactivity (~80%) was attached to oxytocin receptors (high-affinity binding sites). In addition, estimation of the K_d value deduced from the determination of the rate constants K_{+1} and K_{-1} (see legend to Figure 1) was of the same order of magnitude as that determined from dose-dependent binding at equilibrium (0.6 nM compared with 1.8 nM).

A straightforward argument favouring the conclusion that two distinct populations of receptors for oxytocin and vasopressin are present on rat hippocampal synaptosomal membranes was the demonstration that these receptors have clearly different ligand specificities. Thus: (i) OH-Thr^{4}Gly^{7}OT recognized as a highly selective oxytocin agonist on the rat uterus and mammary gland exhibited a high affinity for hippocampal oxytocin receptors and a very low affinity for vasopressin receptors; (ii) Phe^{2}Orn^{8}VT, a selective vasopressin agonist, has an almost 10-times higher affinity for vasopressin receptors than for oxytocin receptors, (iii) the potent vasopressin antagonist d(CH$_2$)$_5$Tyr(Et)VAVP also discriminated efficiently between vasopressin and oxytocin receptors.

The present study clearly illustrates the need for more selective labelled ligands and for additional structure-activity relationship studies for the characterization of receptors for neurohypophyseal hormones and related peptides in the brain. Adenylate cyclase experiments failed to reveal any stimulatory

or inhibitory effect of oxytocin and vasopressin while a significant activation by vasoactive intestinal peptide (VIP) could be demonstrated. This suggests that, unlike oxytocin receptor in frog bladder and epithelial cells (Bockaert *et al.*, 1972), but like oxytocin receptors in mammalian uterus (Harbon and Clauser, 1971) and isolated fat cells (Bonne and Cohen, 1975), oxytocin receptors in the hippocampus are not functionally coupled to adenylate cyclase. In addition there was a fairly good agreement between the relative affinities of oxytocin analogues for oxytocin receptors on hippocampal synaptosomal membranes and the corresponding relative activities in increasing the firing rate of non-pyramidal neurones in hippocampal slices (see Table I). It is tempting to consider that the oxytocin receptors detected in the present study are the physiological receptors involved in the electrophysiological responses described by Muhlethaler *et al.* (1983). Considered together the results of Muhlethaler *et al.* (1983) and those reported in the present study constitute the first example in which the binding of neurohypophyseal peptides to synaptosomal membranes can be correlated to a cellular response.

As indicated above, oxytocin receptors in the hippocampus have some similarities with oxytocin receptors from the uterus, namely a high affinity for the selective oxytocin agonist OH-Thr^{4}Gly^{7}OT and a low affinity for the selective vasopressor agonist Phe^{2}Orn^{8}VT. However, it is already clear that marked differences also exist between peripheral and central oxytocin receptors. The most striking difference is the poor ability of central receptors to discriminate between oxytocin, arginine-vasopressin and arginine-vasotocin. Obviously a more complete analysis of the structural requirements for binding to central and peripheral ocytocin receptor is needed to decide if these receptors belong to different subclasses of oxytocin receptors. As for the vasopressin receptors, they show, as previously mentioned (Barberis, 1983), several similarities with the V_1 type of vasopressin receptors present on vascular smooth muscle cells and hepatocytes.

Materials and methods

Materials

The vasopressin and oxytocin analogues used in this study are listed in Table I. Tritiated arginine-vasopressin labelled on the phenylalanyl residue ([^{3}H]Phe^3-arginine-vasopressin) was purchased from New England Nuclear (Boston, MA). Its specific radioactivity was 60–87 Ci/mmol. Tritiated oxytocin labelled on the tyrosyl residue ([^{3}H]Tyr^2-oxytocin) was purchased from Cambridge Research Biochemicals (Cambridge, UK). Its specific radioactivity was 20 Ci/mmol. The radiochemical purity of these labelled peptides was checked by h.p.l.c. on a Beckman ultrasphere ODS reversed-phase column. Elution was performed with a linear 40 min gradient of 10–50% solvent B at 1 ml/min. Solvent A was 0.1% trifluoroacetic acid (pH 3.5) and solvent B, 0.1% trifluoroacetic acid (pH 3.5) in 75% acetonitrile. The collected fraction corresponding to the tritiated peptide was then poured onto an affinity-chromatography column using neurophysin bound to sepharose 4B (Camier *et al.*, 1973).

Other chemicals were obtained from the following sources: unlabelled arginine vasopressin and oxytocin from Bachem (Bubendorf; Switzerland); sucrose for density gradient ultracentrifugation from Merck (Darmstadt); Tris and GTP (sodium salt) from Sigma Chemical Company (St. Louis, MO); creatine kinase and phosphocreatine (disodium salt) from Boehringer (Mannheim); [^{3}H]cAMP (31.5 Ci/mmol) from New England Nuclear (Boston, MA); [α-^{32}P]ATP (40 Ci/mmol) from Amersham (Bucks, UK).

Membrane preparation

Animals used were male Wistar rats (200 g body wt.) purchased from Iffa Credo (Lyon, France). For each experiment the hippocampi from 20–40 rat brains were dissected and immersed in ice-cold 0.32 M sucrose. The tissue was homogenized in a Potter-Elvehjem homogenizer equipped with a Perspex pestle (0.25-mm clearance) according to previously described procedure (Aldridge *et al.*, 1960). A crude synaptosomal fraction was prepared according to Gray and Whittaker (1962) and submitted to an osmotic shock in 5 mM Tris-HCl buffer (pH 8.1) for 30 min at 0°C. A purified synaptic-membrane fraction essentially free of cytoplasmic constituents was prepared according to Jones and Matus (1974) by flotation in a discontinuous sucrose gradient (34, 28.5 and 10%, w/w) and collected at the 34–28.5% interface after centrifugation for 110 min at 60 000 *g* (average relative centrifugal field) in a SW 28 type swinging bucket rotor of a Beckman ultracentrifuge. The membranes were dispersed in 50 mM Tris-HCl (pH 7.4). 5 mM $MgCl_2$, washed and resuspended in the same medium to a concentration of ~4–6 mg protein/ml. For adenylate cyclase assays, membranes were prepared with 10 mM Tris-maleate pH 7.2 present at all steps of the above procedure. The membranes were then resuspended in 2 mM Tris-maleate pH 7.2, 2 mM EGTA. All experiments were performed using freshly prepared membranes.

Binding assay

Binding of [^{3}H]oxytocin and [^{3}H]vasopressin was measured under experimental conditions identical to those used by Barberis (1983). Membranes (200–300 μg protein/assay) were incubated in a final volume of 200 μl of a medium composed of 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin and various amounts of tritiated ligand. Non-specific binding was determined in the presence of 10 μM unlabelled oxytocin or vasopressin. Incubation was performed at 30°C for 20 min. Bound labelled peptide was separated by filtration through Gelman membrane filters (Metricel GA-3; 1.2 μm). Filters were washed three times with 5 ml of chilled (0°C) 10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$. Usually the duration of the filtration did not exceed 20 s. Radioactivity retained on the filter was counted by liquid scintillation spectrometry. All determinations were performed in triplicate.

The binding curves for oxytocin and vasopressin were determined using an increasing amount of labelled peptide. The dissociation constants at equilibrium (K_d) were statistically calculated as indicated in Figure 3. The binding constants for unlabelled oxytocin and analogues were determined from competition experiments. Membranes were incubated in the presence of a constant amount of [^{3}H]-oxytocin (3 nM) or [^{3}H]vasopressin (1 nM) and increasing amounts of unlabelled peptide.

In order to examine the stability of [^{3}H]oxytocin in binding experiments, 3 nM [^{3}H]oxytocin was incubated with hippocampal membranes in a total assay volume of 200 μl for 25 min at 30°C. After incubation, the membranes were pelleted by centrifugation, 10 nmol of pure, unlabelled oxytocin was then added to the supernatant which was immediately analysed by h.p.l.c., as described above.

Adenylate cyclase assay

Membranes (15–25 μg protein/assay) were incubated in a final volume of 50 μl of a medium containing 25 mM Tris-maleate pH 7.2, 10 mM theophylline, 0.25 mM ATP, 1 mM $MgSO_4$, 5 mM creatine phosphate, 0.2 mg/ml creatine kinase, 1 mg/ml bovine serum albumin, 10 μM GTP, [α-^{32}P]ATP 0.5 μCi/assay, [^{3}H]cAMP 2 nCi/assay. The reaction was initiated by adding the membranes under a small volume (10 μl). They were incubated for 15 min at 30°C and the reaction was stopped by addition of 1 ml of a solution containing: 2% SDS, 50 mM Tris-HCl (pH 7.4), 0.5 mM cAMP and 0.33 mM ATP. Labelled cAMP formed was determined as described (Lucas and Bockaert, 1977). All determinations were performed in triplicate.

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