[^{3}H]Vasopressin binding to rat hippocampal synaptic plasma membrane

Kinetic and pharmacological characterization

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Characterization of specific vasopressin binding sites to rat hippocampal membranes has been assayed using tritiated lysine-vasopressin labelled on the tyrosyl residue. At 30°C specific [^{3}H]vasopressin binding was saturable. The estimated equilibrium dissociation constant was 7.1 nM, the mean maximal binding capacity was 78 fmol/mg protein. Arginine-vasopressin has a high affinity (K_d = 2.8 nM) and dDAVP has a low affinity (K_d = 249 nM) for hippocampal synaptic membranes. (OH)AVP and Phe^{2}Orn^{8}VT are at least as active as AVP in inhibiting [^{3}H]vasopressin binding. Adenylate cyclase was acitvated by VIP and inhibited by PIA, but not affected by lysine-vasopressin.

Synaptic membrane *Hippocampus* *Vasopressin binding* *Adenylate cyclase*

1. INTRODUCTION

Vasopressin and oxytocin are not only neurohypophyseal hormones carried by a vascular route to affect peripheral target tissues but they may also have neural functions. This view is based on:

(1) The demonstration of their presence in synaptic endings of extrahypophyseal pathways [1];
(2) The existence of a calcium-dependent, depolarization-induced release of oxytocin and vasopressin from brain slices [2];
(3) The observations that neurohypophyseal peptides have several behavioral effects including an influence on the retention of conditioned avoidance tasks in rats (review [3];
(4) The demonstration of several (both excitatory and inhibitory) electrophysiological effects of applied oxytocin and vasopressin [4].

Binding studies for neurohypophyseal peptides in the central nervous system have not yet been reported. Such studies would be of importance for the analysis of structure–binding relationships and the design of pharmacological tools which could be very helpful for elucidating the still poorly understood role of neurohypophyseal peptides on brain function [5].

We report the characterization of specific vasopressin binding sites in the rat hippocampus. The choice of this brain region was dictated by:

(1) Oxytocin and vasopressin containing fibers being identified in this region [6];
(2) Hippocampus might be a possible site of action for vasopressin acting on memory processes;
(3) Hippocampal neurones respond to applied vasopressin and oxytocin [4].

2. MATERIALS AND METHODS

2.1. *Synaptosomal membrane preparation*

Animals used were male Wistar rats (200 g body wt) purchased from IFFA CREDO (Lyon). For each experiment the hippocampi from 20 rat brains were dissected and immersed in ice-cold 0.32 M sucrose. The tissue (1.7 g) was homogenized in a Potter homogenizer equipped with a perspex pestle

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(0.25 mm clearance) as in [7].

Synaptic plasma membranes were prepared as in [8]. Briefly a crude synaptosomal fraction was prepared and submitted to an osmotic shock in 5 mM Tris–HCl buffer (pH 8.1) for 30 min at 0°C. Membranes were separated by flotation in a discontinuous sucrose gradient (34%, 28.5% and 10%, w/w) and collected at the 34%–28.5% interface after centrifugation at 60000 × *g* for 110 min. 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 about 2 mg protein/ml. For some adenylate cyclase assays membranes were prepared with EGTA, 1 mM present at all steps of the above procedure. All experiments were performed using freshly prepared membranes.

2.2. *[^{3}H]Vasopressin binding assay*

[*lysine*-^{3}H]Vasopressin labelled on the tyrosyl residue in position 2 was prepared as in [9] and purified by affinity chromatography on a neurophysin–Sepharose column [10]. Radiochemical purity was checked by high-pressure liquid chromatography on a Beckman ultrasphere ODS reversed-phase column. Elution was performed with a linear 40 min gradient of 10–50% B at 1 ml/min. Solvent A was 0.1% trifluoroacetic acid (pH 3.5) and solvent B 0.1% trifluoroacetic acid in 75% acetonitrile. The purified peptide had a specific radioactivity of 8.5 Ci/mM and biological activities undistinguishable from those of the purified starting material (synthetic lysine-vasopressin from Bachem).

Membranes (50–150 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 bovine serum albumin/ml and various amounts of [^{3}H]vasopressin. Non-specific binding was determined in the presence of 10 μM unlabelled lysine-vasopressin. Incubation was performed at 30°C for 20 min. Bound labelled vasopressin was separated by filtration through Gelman membrane filters (Metricel GA-3; 1.2 μm). Filters were washed 3 times by 5 ml 10 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$. Radioactivity retained on the filter was counted by liquid scintillation spectrometry. All determinations were performed in triplicates.

The dissociation constants for unlabelled vasopressin and analogues were deduced from the determination of the dose-dependent inhibition of [^{3}H]vasopressin (10 nM) binding.

Control experiments indicated that:

(1) [^{3}H]Vasopressin was not inactivated during the course of incubation in the presence of membranes (inactivation was checked by HPLC analysis);
(2) The specific and non-specific components of [^{3}H]vasopressin binding increased linearly with up to 300 μg membrane protein/assay;
(3) At 10 nM, a value close to the observed dissociation constant, the non-specific component of [^{3}H]vasopressin binding represented 54 ± 8% of total binding (9 expt);
(4) Freezing the membranes in liquid nitrogen resulted in a 50% decrease in vasopressin binding capacity.

2.3. *Adenylate cyclase assay*

The adenylate cyclase activity of the hippocampal membranes was determined as follows: membranes (30 μg protein/assay) were incubated in a final volume of 100 μl of a medium containing 50 mM Tris–HCl (pH 7.4), 0.5 mM $MgCl_2$, 0.25 mM ATP, 1 mM cAMP, 0.1 mM papaverine, 0.08 IU adenosine deaminase/ml, 1.5 mg creatine phosphate/ml, 1 mg creatine kinase/ml, 1 mg bovine serum albumin/ml, [^{32}P]ATP 0.5 μCi/assay, c[^{3}H]AMP 2 nCi/assay. Determinations were performed in the presence and absence of 0.1 mM GTP and 0.1 mM GTP + 0.2 M NaCl. The membranes were incubated for 10 min at 30°C. The reaction was initiated by adding [^{32}P]-ATP under a small volume (10 μl) and stopped 5 min later by addition of 1 ml of a solution containing: 2% sodium dodecyl sulfate, 50 mM Tris–HCl (pH 7.4), 5×10^{-4} M cAMP, 3.3×10^{-4} M ATP.

2.4. *Peptides used*

The formulae and biological activities of the vasopressin structural analogues used are indicated in table 1.

3. RESULTS AND DISCUSSION

Specific [^{3}H]vasopressin binding to rat hippocampal synaptic plasma membranes was a fairly slow process. At 30°C and for 10 nM ligand, a

Table 1

Affinity constants for binding of vasopressin and analogues to hippocampal synaptic plasma membranes: relation to antidiuretic and vasopressor activities

Peptide	Relative biological activities[a]		Binding to hippocampal membrane	
	Antidiuretic[b]	Vasopressor[b]	K_d (nM)[d]	Relative affinity[a]
Arginine vasopressin (AVP)	100	100	2.8	100
Lysine vasopressin (LVP)	88	73	7.1	40
Oxytocin (OXY)	1.3	1.1	45.3	6
1-(L-2-hydroxy-3-mercapto-propanoic acid)-Arginine vasopressin ((OH) AVP)	146	149	0.9	313
[2-phenylalanine, 8-ornithine]-Vasotocin (Phe^2Orn^8VT)	0.17	33	5.9	48
1-Deamino[8-D-arginine]-vasopressin (dDAVP)	371	0.1	249	1
[1-(β-mercapto-β,β-cyclopentamethylene propionic acid), 2-O-ethyltyrosine, 4-valine]-Vasopressin ($d(CH_2)_5Tyr(et)VAVP$)	Antagonist A2 = 45 nM[c]	Antagonist A2 = 7 nM[c]	3.6	79
[1-(β-mercapto-β,β-cyclopentamethylene propionic acid), 2-O-ethyltyrosine, 4-valine, 8-D-arginine]-Vasopressin ($d(CH_2)_5Tyr(et)VDAVP$	Antagonist A2 = 79 nM[c]	Antagonist A2 = 5 nM[c]	29	10

[a] Percent of AVP taken as a standard

[b] Values used for the calculation of relative vasopressor and antidiuretic activities were taken from [13]

[c] A2 is the antagonist concentration leading the response to 2 × X concentration of agonist to equal that of 1 × X agonist concentration

[d] The dissociation constants for the unlabelled peptides (K_d) was deduced from I_{50}-values; i.e., concentrations of unlabelled peptide leading to half-maximal inhibition of [^{3}H]vasopressin-specific binding, using the following relation $K_d = I_{50} \times K_d[^3H]LVP/(K_d[^3H]LVP) + ([^3H]LVP)$, in which $K_d[^3H]LVP$ is the dissociation constant for [^{3}H]vasopressin binding and ([^{3}H]LVP) the concentration of [^{3}H]vasopressin in the incubation medium

value close to the equilibrium dissociation constant, a maximum value was reached within 15 min and was stable thereafter up to 40 min. The estimated half-time was about 5 min. The corresponding value at 20°C was 8 min. All further experiments were performed at 30°C and the duration of membrane incubation in the presence of [^{3}H]vasopressin was 20 min.

Specific [^{3}H]vasopressin binding was saturable. The results shown in fig.1 indicate that dose-dependent binding was observable in a concentration range covering about two orders of magnitude. The Scatchard plot of the dose–binding curve did not show a clear deviation from linearity suggesting that vasopressin binds to an apparently homogeneous population of specific binding sites. The estimated equilibrium dissociation constant was 7.1 nM, a value which is close to those determined for vasopressin receptors characterized in peripheral organs (review [11]).

The mean maximal binding capacity of 78 fmol/mg protein is lower than those found for

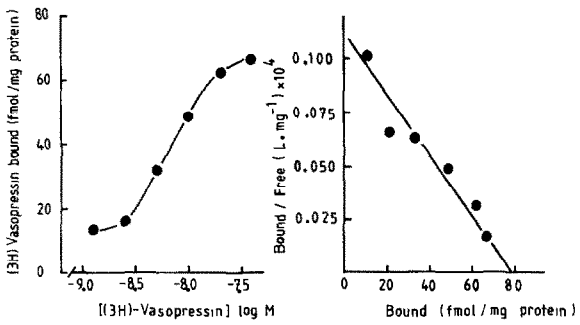

Fig.1. Dose-dependency for specific [^{3}H]vasopressin binding to purified hippocampal synaptic plasma membranes. The binding assay was conducted as in section 2. Left: Specific [^{3}H]vasopressin binding is plotted as a function of the [^{3}H]vasopressin concentration in the incubation medium (log-scale). Values on the graphs are means of 3 independent experiments. Right: Scatchard plots of dose–binding curves shown in the left panel. Values of the dissociation constants and maximal binding capacities estimated from the calculated regression lines are 7.1 nM and 78 fmol/mg protein, respectively.

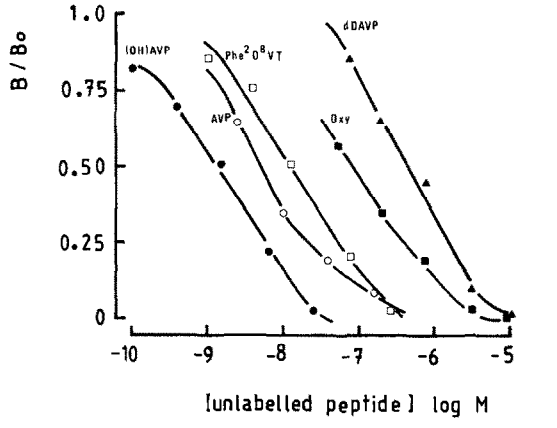

Fig.2. Dose-dependent inhibition of [^{3}H]vasopressin binding to hippocampal synaptic plasma membranes by unlabelled peptides. Membranes were incubated in the presence of a constant amount of [^{3}H]vasopressin (10 nM) and increasing amounts of unlabelled peptides. 5 different concentrations of unlabelled peptides were tested. 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 (B_o). Each point is the mean of triplicate determinations.

kidney or hepatic membranes. This could indicate that vasopressin receptors are not expressed by all hippocampal neurones. Glial localization is unlikely. Indeed the method used for synaptic plasma membrane preparation exclude the larger part of glial elements.

The detected vasopressin binding sites exhibited a high degree of specificity. Unlabelled vasopressin and the vasopressin structural analogues tested inhibited [^{3}H]vasopressin binding to almost the same maximal extent but exhibited marked differences in efficiency. From the data in fig.2 and table 1 it can be concluded that:

(1) Arginine-vasopressin is more efficient than lysine-vasopressin (K_d estimated to 2.8 nM as compared to 7.1 nM for lysine-vasopressin);
(2) dDAVP known as a selective antidiuretic peptide has a low affinity for hippocampal synaptic membranes;
(3) The two analogues (OH) AVP and Phe^2–Orn^8–VT which exhibit a high vasopressor/antidiuretic activities ratio are almost as active (Phe^{2}Orn^{8}VT) or more active ((OH) AVP) than AVP in inhibiting [^{3}H]vasopressin binding.

For the entire series of agonist tested there was a fairly good correspondence between their relative vasopressor activities and their relative affinities for hippocampal membranes.

The two vasopressin antagonists tested were able to inhibit [^{3}H]vasopressin binding to hippocampal membranes. For d(CH$_2$)$_5$Tyr(et)VAVP the apparent dissociation constant is closer to the A2 value for the inhibition of the vasopressor response in vivo than to the corresponding value for the inhibition of the antidiuretic response. For d(CH$_2$)$_5$Tyr(et)VDAVP the estimated dissociation constant appeared to be half-way between the A2 values for the inhibition of the antidiuretic and vasopressor responses. The dissociation constants for the binding of the two antagonists tested were determined on liver membranes which were shown to contain vasopressin receptors of the V_1 (vasopressor) type. The results obtained (not shown) indicated that the affinity of d(CH$_2$)$_5$Tyr(et)VDAVP was about 10 times less than the affinity of d(CH$_2$)$_5$Tyr(et)VAVP. This figure compares very well with the results obtained on rat hippocampal membranes (see table 1).

These data indicate that the recognition pattern

Table 2

Adenylate cyclase activity in hippocampal synaptic plasma membranes

Additions	Control membranes		EGTA-treated membranes	
	+ GTP 0.1 mM	+ GTP 0.1 mM + NaCl 0.2 M	Without GTP and NaCl	+ GTP 0.1 mM + NaCl 0.2 M
None	349 ± 23	334 ± 53	101 ± 16	157 ± 12
VIP 1 μM	485 ± 8[a]	462 ± 21[a]	–	–
PIA 5 nM	–	–	104 ± 6 NS	153 ± 9 NS
50 nM	340 ± 11 NS	281 ± 2 NS	102 ± 6 NS	128 ± 8[a]
5 μM	319 ± 17 NS	280 ± 6 NS	103 ± 7 NS	112 ± 9[a]
LVP 10 nM	–	–	97 ± 9 NS	169 ± 6 NS
100 nM	–	–	99 ± 7 NS	165 ± 4 NS
10 μM	384 ± 25 NS	357 ± 6 NS	100 ± 6 NS	162 ± 8 NS

Values in the table are means ± SD of 4–5 determinations. Adenylate cyclase activities are expressed in pmol cyclic AMP formed.min^{-1}.mg $protein^{-1}$. Values determined in the presence of vasoactive intestinal polypeptide (VIP), phenylisopropyladenosine (PIA) or lysine vasopressin (LVP) were compared to the corresponding basal values using Student's t-test (NS, non-significant; [a] $p < 0.05$)

of hippocampal vasopressin binding sites exhibits marked similarities with that of the so-called V_1 type of vasopressin receptors present on vascular smooth muscle cells and hepatocytes.

The results from the adenylate cyclase experiments are summarized in table 2. Under experimental conditions where an activation of enzyme activity by vasoactive intestinal polypeptide (membranes incubated in the presence of GTP) and inhibition by phenylisopropyladenosine (EGTA-treated membranes incubated in the presence of GTP plus NaCl) could be demonstrated, vasopressin did not change adenylate cyclase activity. This suggests that vasopressin receptors in the hippocampus, unlike renal V_2 receptors but like V_1 vascular and hepatic receptors, are not functionally coupled to adenylate cyclase.

This study shows the existence in the rat hippocampus of specific vasopressin binding sites which have several similarities with previously characterized V_1 vasopressin receptors. They might represent physiological receptors responsible for vasopressin actions in the hippocampus.

A study which demonstrated a close correlation between the vasopressor effects of vasopressin structural analogues and their effects on memory consolidation [12], led to the conclusion that vasopressin might influence avoidance behaviour through its effects on blood pressure. If the detected hippocampal vasopressin receptors, resembling the vasopressin receptors from blood vessels, are those which might be involved in these, still controversial, effects of vasopressin on memory, then our results would indicate that one can hardly argue from investigations such as those conducted in [12] that vasopressin, influences behaviour through a peripheral action.

Furthermore, our results might indicate that both vasopressin and oxytocin receptors are present in the hippocampus. Indeed recent pharmacological studies [4] clearly indicated that the receptors involved in the increased firing rate of hippocampal interneurones have a specificity very close to that of uterine oxytocin receptors.

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REFERENCES

[1] Buijs, R.M. and Swaab, D.F. (1979) Cell Tiss. Res. 204, 353–365.
[2] Buijs, R.M. and Van Heerikhuize, J.J. (1982) Brain Res. 252, 71–76.
[3] De Wied, D. (1980) Proc. R. Soc. London B210, 183–195.
[4] Mühlethaler, M., Dreifuss, J.J. and Gähwiler, B.H. (1982) Nature 296, 749–751.
[5] Gash, D.M. and Thomas, G.J. (1983) Trends Neurosci. 6, 197–198.
[6] Buijs, R.M. (1978) Cell Tiss. Res. 192, 423–453.
[7] Aldridge, W.N., Emery, R.C. and Street, B.W. (1960) Biochem. J. 77, 326–327.
[8] Jones, D.H. and Matus, A.I. (1974) Biochim. Biophys. Acta 356, 276–287.
[9] Pradelles, P., Morgat, J.L., Fromageot, P., Camier, M., Bonne, D., Cohen, P., Bockaert, J. and Jard, S. (1972) FEBS Lett. 26, 189–192.
[10] Camier, M., Alazard, R., Cohen, P., Pradelles, P., Morgat, J.L. and Fromageot, P. (1973) Eur. J. Biochem. 32, 207–214.
[11] Jard, S. (1983) in: Current Topics in Membranes and Transport (Kleinzeller, A. ed) vol.18, pp.255–285, Academic Press, New York.
[12] Le Moal, M., Koob, G.F., Koda, L.Y., Bloom, F.E., Manning, M., Sawyer, W.H. and Rivier, J. (1981) Nature 291, 491–493.
[13] Manning, M., Gzronka, Z. and Sawyer, W.H. (1981) in: The Pituitary (Beardwell, C. and Robinson, G. eds) pp.265–296, Butterworths, London.