Determination of the functional molecular size of vasopressin isoreceptors

P. Crause, R. Boer and F. Fahrenholz*

Max-Planck-Institut für Biophysik, Kennedyallee 70, D-6000 Frankfurt, FRG

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The molecular size of vasopressin receptors in the intact membrane-bound state was determined by radiation inactivation (target size analysis). For the V_1 receptor in rat liver a molecular size of (76±8) kDa was determined. For the V_2 receptor in rat kidney and bovine kidney molecular sizes of (95±4) and (108±11) kDa were found. Statistical analysis gave evidence for size differences between rat liver and rat kidney receptors or differences between rat liver and bovine kidney receptors, but not between kidney receptors from different species. The results suggest that V_1 and V_2 receptors can be distinguished by functional properties as well as by their size.

Vasopressin receptor *Radiation inactivation* *Target size* *Plasma membrane*

1. INTRODUCTION

Among the variety of physiological effects shown by the neurohypophyseal peptide hormone vasopressin, its antidiuretic action in the mammalian kidney and its glycogenolytic activity in the liver have been extensively studied. Specific receptors for vasopressin have been identified in these target tissues, and biochemical analysis revealed different mechanisms of signal transfer [1]. On the one hand, it is well known that the interaction of vasopressin with its receptor in the mammalian kidney activates the enzyme adenylate cyclase. On the other hand, there is recent evidence that the binding of vasopressin to its receptor in the rat liver induces the mobilization of intracellular Ca^{2+} with myo-inositol 1,4,5-triphosphate as second messenger [2]. Studies with structural analogs of the natural hormone have clearly shown different recognition patterns of vasopressin receptors in kidney and liver [1,3]. Therefore, mammalian vasopressin receptors from liver and kidney are now generally classified as V_1 and V_2 receptors [4].

To examine whether the functional difference between the two types of vasopressin receptors (vasopressin isoreceptors [1]) reflects a difference in molecular size, we determined this structural parameter of the V_1 and V_2 receptors in the membrane-bound state by radiation inactivation (target size analysis). This technique gives the functional size of a protein complex, rather than the molecular masses of individual polypeptides [5]. Knowledge of the functional molecular size of vasopressin receptors would also be valuable for the interpretation of affinity labeling studies.

2. EXPERIMENTAL

2.1. *Plasma membrane preparations*

Plasma membranes from bovine kidney medulla and rat liver were prepared as described previously [3,6]. For the preparation of plasma membranes from rat kidney medulla the following procedure was applied: tissue derived from the medulla of rat kidneys was suspended in 0.9% NaCl, 1 mM EDTA (pH 7.4) and passed through a stainless steel tissue press. Particulate material was collected by centrifugation at 2000 × g_{max} for 5 min and resuspended in 0.25 M sucrose, 1 mM EDTA and

* To whom correspondence should be addressed

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5 mM Hepps (pH 7.8) at a ratio of 5 ml medium per g medullary tissue. It was subsequently homogenized with a dounce homogenizer performing 10 strokes with the loose pestle and 3 strokes with the tight one. The suspension was filtered through 3 layers of gauze and subsequently centrifuged at $600 \times g_{max}$ for 10 min. The supernatant was recentrifuged at $50000 \times g_{max}$ for 20 min, yielding a hard brown pellet and a fluffy layer. The latter was separated and further fractionated by Percoll density gradient centrifugation following the same protocol as described for the bovine kidney preparation [6]. By this method partially purified plasma membranes from rat kidney medulla were obtained containing 0.8 pmol specific vasopressin binding sites per mg protein. Before irradiation, the membranes were washed free from sucrose by repeated pelleting and resuspension in 20 mM Hepps (pH 8.3). The final suspension was fractionated into polypropylene cups (300–500 μl) and kept at −80°C.

2.2. *Irradiation procedures*

10 MeV electrons were generated by a linear accelerator (CSF Thompson, Paris) at dose rates of 2–3 Mrad/min. Samples were kept at −85°C during irradiation using the sample holder described in [7], which was cooled by a stream of liquid nitrogen. To determine doses of radiation, radiochromic dye films (Far-West Technology, Goleta, CA) were inserted into separate positions of the sample holder. The radiation dose was estimated as described [7] and plotted against the position in the sample holder yielding a calibration curve.

2.3. *Assays*

The inactivation of vasopressin receptors was determined by measuring the decay of specific binding capacity for [^{3}H][Arg^8]vasopressin ([^{3}H]-AVP). The radioligand was either prepared with a specific radioactivity of 6.6 Ci/mmol [6] or obtained from New England Nuclear (47 Ci/mmol). All binding assays were performed in triplicate, using the filtration method as described [3]. Receptor density was determined as the specific binding at a total concentration of 10^{-8} M [^{3}H]AVP after 30 min incubation at 30°C. This concentration leads to more than 90% saturation of receptor sites. As protein standards for the radiation inactivation horse liver alcohol dehydrogenase (Boehringer, Mannheim, FRG) and the endogenous alkaline phosphatase of rat liver plasma membranes were used. Enzyme activity was determined by photometric assays [8,9].

2.4. *Data presentation and calculations*

For each irradiated sample the logarithm of binding capacity or enzyme activity with standard deviation (SD) was calculated and the corresponding radiation dose was obtained from the calibration curve. In all experiments a linear relationship was found as analysed by a weighted linear least squares regression. Doses yielding either 37% residual binding capacity or enzyme activity (D_{37}) were calculated from the equations describing the regression lines. From the average $D_{37} \pm$ SD derived from several separate experiments, the molecular size (in Da) of the corresponding receptor or standard enzyme was calculated using the empirically established equation [10]

$$\text{molecular size} = f \times \frac{6.4 \times 10^5}{D_{37}},$$

where D_{37} is in Mrad and $f = 2.1$ is a temperature correction factor derived from [11]. For the statistical analysis of target size differences, Student's t-test was applied.

3. RESULTS AND DISCUSSION

The binding of ligands to membrane-bound receptors is characterized by two parameters: the maximal capacity reflecting the density of specific binding sites in the membrane and the binding affinity, which is usually expressed as the dissociation constant K_D. In accordance with the concept that one single hit with high energy electrons inactivates one target molecule [5], it has been shown for several membrane-bound receptors that irradiation with high energy electrons reduces the number of binding sites but not the binding affinity [12–14]. To ensure that this assumption is also valid for vasopressin receptors the saturation isotherms of specific [^{3}H]AVP-binding to irradiated and nonirradiated rat liver membranes were analysed. Fig.1 and table 1 demonstrate that the high energy radiation used in this study reduces the number of vasopressin receptor sites without influencing the binding affinity.

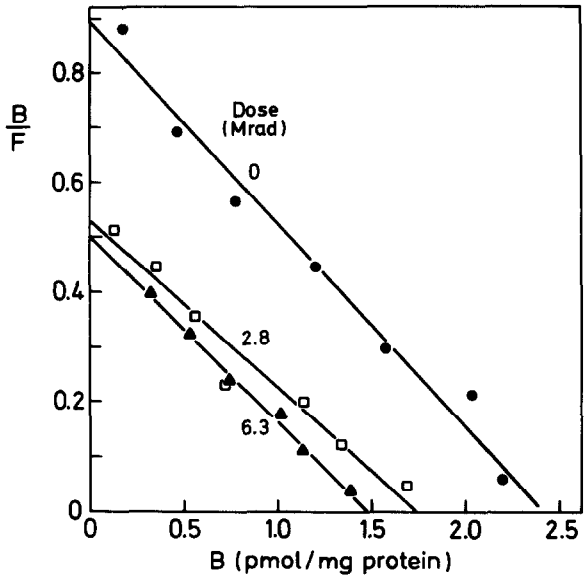

Fig.1. Scatchard analysis of saturation isotherms of specific [^{3}H]AVP-binding to rat liver plasma membranes before (●) and after irradiation (□, 2.8 Mrad; ▲, 6.3 Mrad).

For the plasma membranes isolated from bovine kidney, rat kidney and rat liver, linear inactivation plots of the specific binding sites were obtained (fig.2), indicating the presence of homogenous species of vasopressin receptors. Our finding confirms the results of binding studies, which demonstrated non-cooperative binding of vasopressin to homogenous sites in those membranes [1].

The functional molecular sizes of vasopressin isoreceptors in the membrane-bound state and of the standard enzymes calculated from the average

Table 1

Dissociation constants K_D for the specific binding of [^{3}H]AVP to irradiated and nonirradiated plasma membranes from rat liver

Irradiation dose (Mrad)	K_D (M)
0	$(6.9 \pm 1.2) \times 10^{-10}$
2.8	$(8.4 \pm 2.2) \times 10^{-10}$
6.3	$(7.6 \pm 0.9) \times 10^{-10}$

The K_D values were calculated from the slopes of the regression lines presented in fig.1. Error estimates are expressed as 95% confidence interval

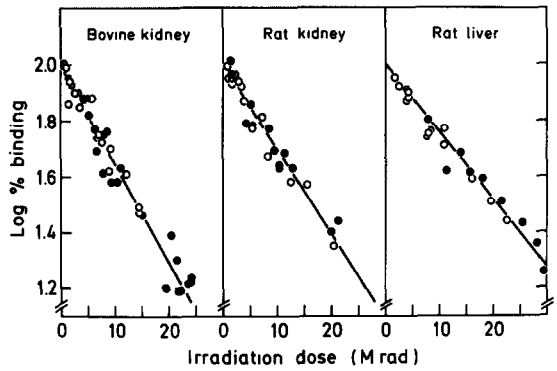

Fig.2. Inactivation plots of specific [^{3}H]AVP-binding sites in plasma membranes from bovine kidney, rat kidney and rat liver. For graphic representation, the data from the two experiments which were closest to the average D_{37} value were combined.

D_{37} values are given in table 2. The good agreement of the known molecular mass of standard enzymes with the functional molecular size determined by target size analysis in our study, demonstrates the validity of the method. For the V_1 receptor in rat liver (76 ± 8) kDa was determined, for the V_2 receptor in rat kidney and bovine kidney (95 ± 4) and (108 ± 11) kDa, respectively, were found. For comparison, by hydrodynamic methods applied to solubilized hormone-receptor complexes, molecular masses of 83 and 80 kDa for V_1 and V_2 receptors from rat liver and rat kidney have been determined [15].

Taking into account the experimental errors in both the target size analysis and the semi-empirical hydrodynamic method, the molecular size of vasopressin isoreceptors in the membrane-bound state is roughly the same as in detergent solution. However, in contrast to the results obtained by the hydrodynamic method [15], statistical analysis of our data reveals a significant size difference between the hepatic V_1 and the renal V_2 receptors of the rat ($p < 0.01$). The evidence for a size difference between liver and kidney receptors is even greater if the values of vasopressin receptors from bovine kidney and the rat liver are compared ($p < 0.001$). In contrast, no significance could be found for a size difference between the V_2 receptors from rat and bovine kidney ($p > 0.05$).

In conclusion, the results of the present study suggest that the vasopressin receptors from mammalian liver and kidney differ not only in their

Table 2

Molecular sizes of vasopressin receptors and standard enzymes

Target	n	D_{37} (Mrad)	Molecular size (kDa)	
			Target size	Reference
Vasopressin receptor (rat liver)	9	17.8 ± 1.9	76 ± 8	83[a]
Vasopressin receptor (rat kidney)	3	14.2 ± 0.6	95 ± 4	80[a]
Vasopressin receptor (bovine kidney)	7	12.5 ± 1.3	108 ± 11	–
Alcohol dehydrogenase (horse liver)	9	15.7 ± 2.2	86 ± 12	80[b]
Alkaline phosphatase (rat liver)	10	18.7 ± 2.3	72 ± 9	70[c] 75[d]

[a] Based on the hydrodynamic characterization of solubilized hormone-receptor complexes [15]

[b] Molecular mass of the dimeric enzyme [16]

[c] Target size of the subunit when irradiated in the absence of K^+-stimulation [10]

[d] Subunit size as determined by SDS–PAGE [9]

The molecular sizes were calculated from the average D_{37} values as described in section 2. For comparison molecular size reference values are given

functional properties, but also in molecular size. Therefore, the V_1 and V_2 receptors found in mammalian tissues may represent products of different genes.

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REFERENCES

[1] Jard, S. (1983) Curr. Top. Membr. Transp. 18, 255–285.

[2] Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F. and Williamson, J.R. (1984) J. Biol. Chem. 259, 3077–3081.

[3] Fahrenholz, F., Boer, R., Crause, P., Fritzsch, G. and Grzonka, Z. (1984) Eur. J. Pharmacol. 100, 47–58.

[4] Michell, R.H., Kirk, C.J. and Billah, M.M. (1979) Biochem. Soc. Trans. 7, 861–865.

[5] Kempner, E.S. and Schlegel, W. (1979) Anal. Biochem. 92, 2–10.

[6] Crause, P. and Fahrenholz, F. (1982) Mol. Cell. Endocrinol. 28, 529–541.

[7] Ferry, D.R., Goll, A. and Glossmann, H. (1983) Naunyn-Schmiedeberg's Arch. 323, 292–297.

[8] Oppenheimer, H.L., Green, R.W. and McKay, R.H. (1967) Arch. Biochem. 119, 552–559.

[9] Ohkubo, A., Langerman, N. and Kaplan, M.M. (1974) J. Biol. Chem. 249, 7174–7180.

[10] Kepner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta 163, 188–203.

[11] Kempner, E.S. and Haigler, H.T. (1982) J. Biol. Chem. 257, 13297–13299.

[12] Chang, L.-R. and Barnard, E.A. (1982) J. Neurochem. 39, 1507–1518.

[13] Fraser, C.M. and Venter, J.V. (1982) Biochem. Biophys. Res. Commun. 109, 21–29.

[14] Steer, C.J., Kempner, E.S. and Ashwell, G. (1981) J. Biol. Chem. 256, 5851–5856.

[15] Guillon, G., Couraud, P.-O., Butlen, D., Cantau, B. and Jard, S. (1980) Eur. J. Biochem. 111, 287–294.

[16] Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T. and Bränden, C.-I. (1974) FEBS Lett. 44, 200–204.