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Identification of the V_1 Vasopressin Receptor by Chemical Cross-Linking and Ligand Affinity Blotting†

Enrique F. Estrada,‡ Valeria Barra,‡ Carlos E. Caorsi,‡ Silvia Troncoso,‡ Nelson Ruiz-Opazo,§ and Carlos B. González*,‡

Instituto de Fisiología, Facultad de Medicina, Universidad Austral de Chile, Valdivia, Chile, and Section of Molecular Genetics, Boston University School of Medicine, Boston, Massachusetts 02118

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ABSTRACT: Chemical and photoaffinity cross-linking experiments as well as ligand affinity blotting techniques were used to label the V_1 vasopressin receptor. In order to determine the optimal reaction conditions, pig liver membranes were incubated with 5 nM [8-lysine]vasopressin (LVP) labeled with ^{125}I and then cross-linked with the use of DMS (dimethyl suberimidate), EGS [ethylene glycol bis(succinimidyl succinate)] or HSAB (hydroxysuccinimidyl *p*-azidobenzoate) at different final concentrations. Consistently, EGS was found to label with high yield one band of M_r 60000 in rat and pig liver membranes when used at a final concentration between 0.05 and 0.25 mM. The protein of M_r 60000 is labeled in a concentration-dependent manner when pig liver membranes are incubated with increasing concentrations of ^{125}I-LVP and then cross-linked with EGS. The label was displaced by increasing concentrations of unlabeled LVP or d(CH_2)$_5$[Tyr^2(Me),-Tyr^9(NH_2)]AVP (V_1/V_2 antagonist). A protein band of similar molecular mass was cross-linked with ^{125}I-LVP in rat liver membranes. The reaction was specific since the incorporation of label into the protein of M_r 60000 was inhibited by LVP, [8-arginine]vasopressin (AVP), the V_1/V_2-antagonist, and the specific V_1-antagonist d(CH_2)$_5$[Tyr^2(Me)]AVP, only partially by [des-Gly^9]AVP (V_2-agonist) and by oxytocin, and not at all by angiotensin II. Incubation of nitrocellulose containing membrane proteins from pig liver with ^{125}I-LVP showed the labeling of a band of M_r 58000 that is inhibited by an excess of unlabeled LVP. This band of M_r 58000 seems to correspond with the protein of M_r 60000 revealed by the cross-linking experiment. This protein appears not to have internal disulfide bonds since the electrophoretic pattern did not change in the absence or presence of reductant in the polyacrylamide gel electrophoresis. Our results suggest that the V_1 vasopressin receptor is a monomeric protein of M_r 60000.

Vasopressin, which is secreted into the systemic circulation in the neural lobe (Pickering et al., 1986), exhibits a wide range of biological activities. In addition to antidiuresis and vasoconstriction, these activities include regulation of glucose metabolism (Hems & Whitton, 1973; Cantau et al., 1980), platelet aggregation (Haslam & Rosson, 1972), regulation of the motility of epididymis and vas deferens (Jaakkola & Talo, 1981), and modulation of neuronal function (De Wied et al., 1978). These diverse biological effects are produced by the interaction of the hormone with at least two types of receptors (Michell et al., 1979). The V_1 type receptor stimulates the formation of 1,2-diacylglycerol and inositol 1,3,4-triphosphate, thus increasing cytosolic Ca^{2+}, whereas the V_2 type receptor activates the formation of cyclic AMP (De Wulf et al., 1980; Jard, 1983a; Guillon et al., 1986). The V_1 type receptor has been identified in the liver (Cantau et al., 1980), in the vascular smooth muscle (Penit et al., 1983), in platelets (Siess et al., 1986), in some portions of the male genital tract (Maggi et al., 1987), and in the brain (Pearlmutter et al., 1983). The V_2 type receptor has been found in the renal medulla (Bockaert et al., 1973) and in the seminal vesicles (Maggi et al., 1988).

An understanding of the events in vasopressin receptor activation and function involves the identification and molecular characterization of the different components of the receptor system.

Conflicting evidence has been reported concerning the molecular structure of the vasopressin receptor. Apparent

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* To whom correspondence should be addressed.
‡ Universidad Austral de Chile.
§ Boston University School of Medicine.

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molecular masses ranging from 140000 to 30000 have been reported by chemical cross-linking or photoaffinity labeling. In rat liver membranes, proteins of M_r 30000 and 38000 have been identified by photoaffinity labeling (Boer & Fahrenholz, 1985; Fahrenholz et al., 1985), whereas in kidney these authors found a major component of M_r 30000 and a minor one of M_r 50000–60000 (Fahrenholz et al., 1985, 1988). In turn, in a pig kidney cell line a major protein of M_r 50000 and a minor one of M_r 30000 have been found with use of covalent cross-linking (Marie & Roy, 1988). In human platelet, a protein of M_r 145000 has been revealed by direct ultraviolet photoaffinity (Thibonnier, 1987). Recently, Aiyar et al. (1989) have reported a single band of M_r 62000 for the pig kidney receptor using photoaffinity labeling.

In this study we identify by cross-linking experiments a protein of M_r 60000 in rat and pig liver membranes as the vasopressin receptor. Additionally, by using specific analogues we showed that this protein is the V_1 type of vasopressin receptor.

Materials and Methods

Membrane Preparation. Fresh pig liver (30 g) was homogenized with a Teflon glass homogenizer in 250 mL of buffer A (10 mM HEPES,[1] pH 7.5, 5 mM $MgCl_2$, 2 mM EGTA, 0.2 mM PMSF) containing 100 μg of leupeptin and of 100 μg of pepstatin. The homogenate was centrifuged for 10 min at 6000 rpm in a Kubota centrifuge in a RA3 rotor. The supernatant was centrifuged for 1.5 h at 17500 rpm. The pellet was resuspended in 5 mL of buffer A, and protein content was determined according to Peterson (1977). In some experiments, rat or pig liver membranes were purified by sucrose gradient centrifugation according to the method described by Neville (1968) except that the homogenization was carried out in 1 mM sodium bicarbonate containing 5 mM $MgCl_2$, 2 mM EGTA, 0.2 mM PMSF, 100 μg of leupeptin, and 100 μg of pepstatin per 250 mL of solution. Membranes were diluted to a final concentration of protein of 10 mg/mL with the same buffer, and sucrose was added to a final concentration of 250 mM. The membranes were sonicated, aliquoted, and stored at −20 °C until use.

Cross-Linking Experiments. Unless otherwise stated, 75 μg of membranes was incubated at room temperature for 40 min with 5 nM ^{125}I-LVP in buffer A in a final volume of 200 μL. Nonspecific binding was determined in the presence of 10 μM LVP. For the saturation experiments, various concentrations of ^{125}I-LVP (0.5–15 nM) were used. Competition experiments were carried out by incubation in the presence of increasing concentrations of LVP or the V_1/V_2-antagonist d(CH_2)$_5$[Tyr^2(Me),Tyr^9(NH_2)]AVP (kindly supplied by Professor M. Manning). Hormone specificity was determined by adding 10 μM either LVP, AVP, V_1/V_2-antagonist, the V_1-antagonist d(CH_2)$_5$[Tyr^2(Me)]AVP, the V_2-agonist [des-Gly^9]AVP, oxytocin, or angiotensin II. After incubation the reaction mixture was placed on ice for 10 min and the cross-linking agent DMS, EGS, or HSAB at varying final concentrations (0.05, 0.25, 0.5, and 1 mM) was added from a freshly prepared 100 mM stock solution. The mixture was incubated for an additional 10 min, and the reaction was quenched by the addition of Tris buffer, pH 8.0. The photoactivated cross-linker HSAB was irradiated for 10 min with a UV lamp at 359 nm.

Iodination. LVP (2 nmol) was incubated with 1 mCi of Na[^{125}I]I in 10 μL of 0.5 M NaH_2PO_4, pH 7.0, in an Iodogen-coated microfuge tube (Fraker & Speck, 1978). After 10 min the reaction was stopped by dilution and then immediately passed through a Sep-Pak cartridge and the peptide was eluted with 2 mL of 80% 2-propanol/0.1% trifluoroacetic acid.

SDS–Polyacrylamide Gel Electrophoresis. Cross-linked membranes were precipitated by the addition of 10% TCA for 1 h on ice and centrifuged at 13000 rpm for 30 min in a microfuge, and the precipitates were resuspended in sample buffer with or without 2-mercaptoethanol. Electrophoresis was carried out in 12.5% acrylamide in a discontinuous buffer system according to the method of Laemmli (1970). Gels were stained and dried, followed by autoradiography with Kodak XA-5 film.

Ligand Affinity Blotting. Membranes proteins were separated by SDS–polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes according to Twonbin et al. (1979). Nitrocellulose strips were blocked with a solution of 1% BSA in phosphate-buffered saline (PBS) and then incubated overnight with 10 nM ^{125}I-LVP in PBS containing 0.3% Tween 20. After incubation the nitrocellulose was washed for at least 3 h with PBS–Tween, dried, and exposed.

Results

Membrane proteins from pig liver were separated by SDS–polyacrylamide gel electrophoresis under reducing conditions, electroblotted onto nitrocellulose membranes, and incubated with ^{125}I-LVP in the presence or absence of an excess of unlabeled LVP. Autoradiograms showed that ^{125}I-LVP binds mainly to a broad band of M_r 58000 and to a minor extent to bands of M_r 64000 and M_r 72000. Neither the relative migration nor the binding capacity of these proteins was altered by omitting or including 2-mercaptoethanol in the sample buffer. Addition of an excess of unlabeled LVP completely blocked the binding of ^{125}I-LVP to membrane proteins transferred onto nitrocellulose filters (Figure 1).

In order to determine the optimal experimental conditions, pig liver membranes incubated to binding equilibrium with ^{125}I-LVP were exposed to increasing concentrations of bifunctional cross-linking agents and then subjected to SDS–polyacrylamide gel electrophoresis and radioactive label visualized by autoradiography. Whem DMS was used as the cross-linking reagent at various concentrations (see Materials and Methods) a major broad band of M_r 56000 and two minor ones of M_r 35000 and M_r 72000 were seen at all concentrations of DMS used (Figure 2). The total amount of radioactive label and the proportion of different bands did not change with the different concentration of this cross-linker. The use of EGS as a cross-linking reagent resulted in the labeling of a single band of M_r 60000 at concentrations of 0.05 and 0.25 mM, whereas at concentrations of 0.5 and 1.0 mM in addition to the M_r 60000 protein another band of M_r 35000 is labeled. The amount of labeling increased slightly from 0.05 to 0.5 mM. In turn, at 1 mM the total amount of labeling decreased, and the relative proportion of the labeling of bands drastically changed (Figure 2). This is probably due to excessive cross-linking of the proteins, since most of the radio-

[1] Abbreviations: LVP, [8-lysine]vasopressin; AVP, [8-arginine]vasopressin; V_1/V_2-antagonist, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*o*-methyl)tyrosine,9-tryosinamide,8-arginine]vasopressin; V_1-antagonist, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(*o*-methyl)tyrosine,8-arginine]vasopressin; V_2-agonist, [des-9-glycine,8-arginine]vasopressin; DMS, dimethyl suberimidate; EGS, ethylene glycol bis(succinimidyl succinate); HSAB, hydroxysuccinimidyl *p*-azidobenzoate; Iodogen, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoril; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

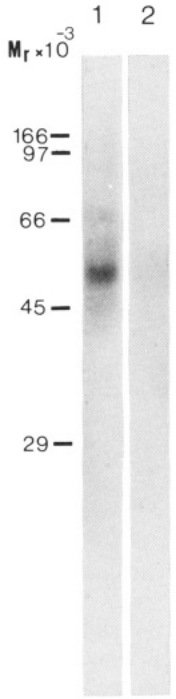

FIGURE 1: Ligand affinity blotting. Proteins from pig liver membranes were separated by polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose, which was incubated with 10 nM ^{125}I-LVP in the absence (lane 1) or in the presence of an excess (10 μM) of cold LVP (lane 2).

active label failed to enter the gel and remained on the top of the stacking gel as aggregated material. Consistently, the photoreactive cross-linking reagent HSAB showed similar results, that is, a major band of M_r 60 000 and two minor ones of M_r 35 000 and M_r 72 000.

Following the detection of consistency in the results between EGS and HSAB as cross-linking agents, subsequent studies were performed with use of only EGS as a cross-linking reagent. To ascertain the specificity with which ^{125}I-LVP recognizes the M_r 60 000 band, various peptide hormones were examined for their ability to displace the labeling of the protein of M_r 60 000 at a concentration of 10 μM; densitometric analyses of respective displacement experiments indicated that LVP, AVP, and the V_1/V_2-antagonist displaced about 90% of the ^{125}I-LVP. In marked contrast, oxytocin displaced about 10% of the ^{125}I-LVP labeling, whereas angiotensin II was unable to inhibit the binding of ^{125}I-LPV to the protein of M_r 60 000 (Figure 3). The electrophoretic migration of the labeled band was not altered by the omission of 2-mercaptoethanol in the sample buffer (Figure 3).

Highly purified pig liver membranes prepared according to the methods described by Neville (1968) showed exactly the same pattern of labeling (Figure 4, lane 1) as the crude membrane preparation.

Cross-linking of ^{125}I-LVP to rat liver membranes resulted in the labeling of a protein band that ran slightly faster than the protein labeled in pig liver membranes. Similarly, the label of the band of M_r 60 000 from rat liver membranes was displaced by LVP and the V_1-antagonist and only slightly by the V_2-agonist (Figure 4).

When an increasing concentration of the labeled ligand was incubated with a fixed amount of pig liver membranes, the cross-linking of the ^{125}I-LVP to the protein of M_r 60 000 increased in a concentration-dependent manner and was a saturable process (Figures 5 and 6).

Competition for specific ^{125}I-LVP affinity cross-linking of pig liver membranes by unlabeled LVP and V_1/V_2-antagonist was examined by incubation of membranes with 5 nM ^{125}I-LVP and an increasing concentration of native hormone or the V_1/V_2-antagonist ranging from $5 \times (10^{-8}$–$10^{-4})$ M. With an increasing concentration of the unlabeled LVP or the V_1/V_2-antagonist, decreased labeling of the band of M_r 60 000 was observed (Figure 7A,B).

Discussion

Several considerations have been taken into account not only to maximize receptor–ligand linkages and to minimize excessive or nonproductive cross-linking but also to specifically label the receptor. (i) Three different cross-linking reagents were tried: two homobifunctional and one heterobifunctional photoactivable cross-linking reagents. (ii) Different proportions of reagent concentration, that is cross-linker and proteins, have been used to prevent unwanted reactions. (iii) The range of

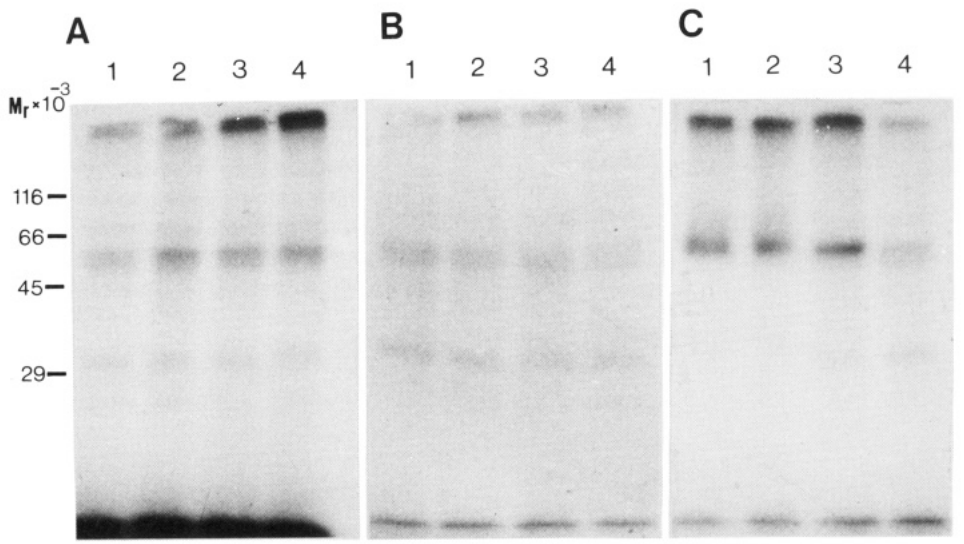

FIGURE 2: Autoradiograms of a polyacrylamide gel of ^{125}I-LVP cross-linked to pig liver membranes using different concentrations of HSAB (panel A) or DMS (panel B) or EGS (panel C). Pig liver membranes were incubated with 5 nM ^{125}I-LVP for 40 min, and then different cross-linking reagents were added to a final concentration of 0.05 mM (lane 1 in panels A, B, and C), 0.25 mM (lane 2 in panels A, B, and C), 0.5 mM (lane 3 in panels A, B, and C), and 1 mM (lane 4 in panels A, B, and C). When HSAB was used as a cross-linker, this was activated by irradiation with a UV lamp. The molecular weight markers correspond to β-galactosidase (116 000), phosphorylase *b* (97 000), BSA (66 000), ovoalbumin (45 000), and carbonic anhydrase (29 000).

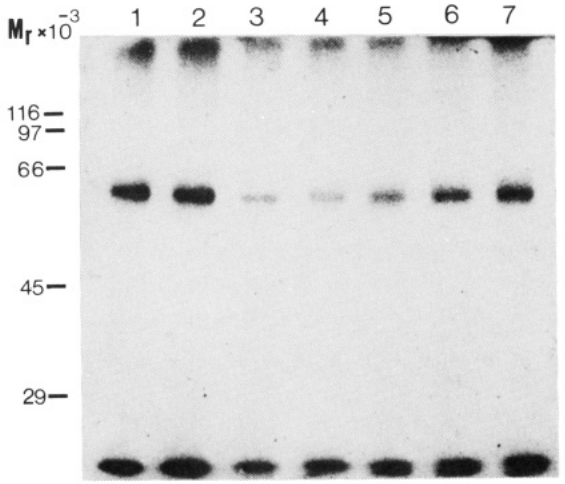

FIGURE 3: Autoradiogram of a polyacrylamide gel of ^{125}I-LVP cross-linked to pig liver membranes in the absence (lane 1 and 2) or in the presence of 10 μM LVP (lane 3), V_1/V_2-antagonist (lane 4), AVP (lane 5), oxytocin (lane 6), and angiotensin II (lane 7). In lane 2, proteins were electrophoresed in the absence of 2-mercaptoethanol.

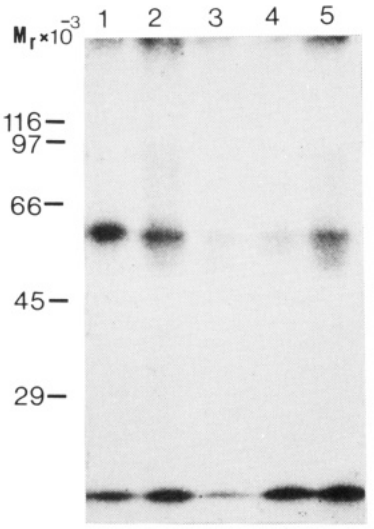

FIGURE 4: Autoradiogram of a polyacrylamide gel of ^{125}I-LVP cross-linked to a highly purified pig liver membrane preparation (lane 1) or to highly purified rat liver membranes (lanes 2, 3, 4, and 5). The reaction was carried out in the presence of 10 μM LVP (lane 3), V_1-antagonist (lane 4), and V_2-agonist (lane 5).

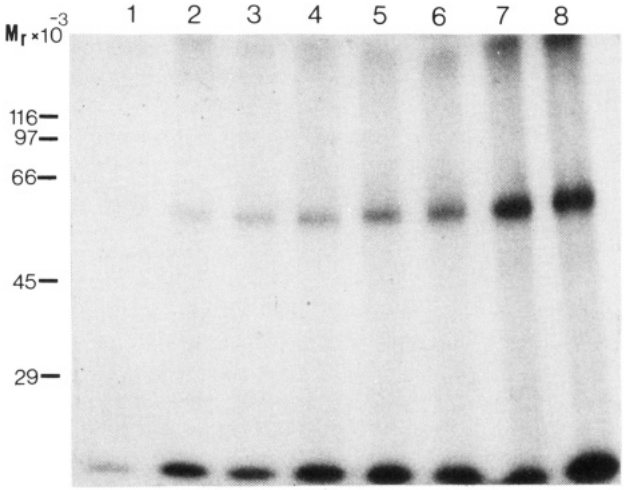

FIGURE 5: Saturation analysis. Liver membranes were incubated with increasing concentrations of ^{125}I-LVP and then cross-linked with use of EGS. Lanes: (1) 1 nM, (2) 2 nM, (3) 3 nM, (4) 4 nM, (5) 5 nM, (6) 7 nM, (7) 10 nM, (8) 15 nM ^{125}I-LVP.

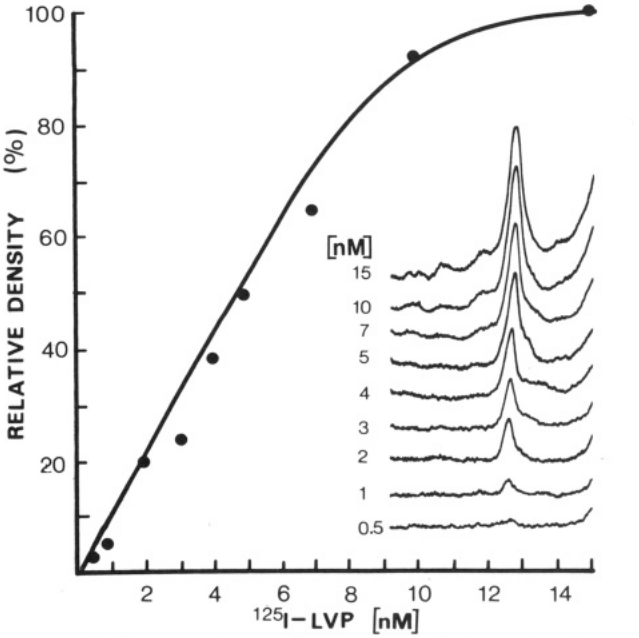

FIGURE 6: Binding curve of saturation experiment similar to that shown in Figure 4. A densitometric analysis (inset) of the autoradiogram of a polyacrylamide gel was carried out, and the area under the peak was integrated and plotted as percentage of the maximal density against the concentration of ^{125}I-LVP added.

concentration of the hormone used was such that only high-affinity sites are shown. (iv) To increase the probability of cross-linking [8-lysine]vasopressin was used as a ligand to provide an extra amino group, thus also favoring the geometry spacing conditions of the amino groups for cross-linking.

Similar results were obtained with use of HSAB and EGS, that is the labeling of a major protein of M_r 60000 and a minor of M_r 35000. When DMS was used as a cross-linker, a similar pattern was observed except that the protein of M_r 60000 ran slightly faster. One explanation for the difference in the apparent molecular mass on SDS gels might be that DMS is producing intramolecular cross-linking preventing the total unfolding of the protein under reducing conditions. Alternatively, the difference might be due to two forms of the receptor. DMS might be cross-linking one form of lower molecular mass whereas HSAB and EGS are labeling the other of higher molecular mass. The difference in the molecular mass might be a result of posttranslational glycosylation or difference in the primary structure of the protein. Many membrane receptors have been shown to be glycosylated (Lomasney et al., 1986; Susuni et al., 1986), and some of them display similar heterogeneity (Nicola & Peterson, 1986; Bruno & Berelowitz, 1989). Interestingly, preliminary experiments have shown that the protein of a M_r of 60000 can be cross-linked after passage of solubilized liver membranes through a wheat germ agglutinin–Sepharose column, suggesting that this protein is glycosylated.

Since the cross-linking of LVP in the nanomolar concentration range to only one band proceeded with high yield when EGS was used at low concentration, this cross-linker was used to try the specificity and saturability of the reaction. The protein of M_r 60000 obtained with EGS displayed the characteristic expected for the vasopressin receptor. Indeed, the radiolabel incorporation into the form of M_r 60000 was proportional to the concentration of the ^{125}I-LVP in the nanomolar range and reached a saturation at concentrations greater than 10 mM. The reaction was inhibited in a concentration-dependent manner by LVP and V_1/V_2-antagonist. The reaction was specific since the label was displaced by LVP, AVP, and

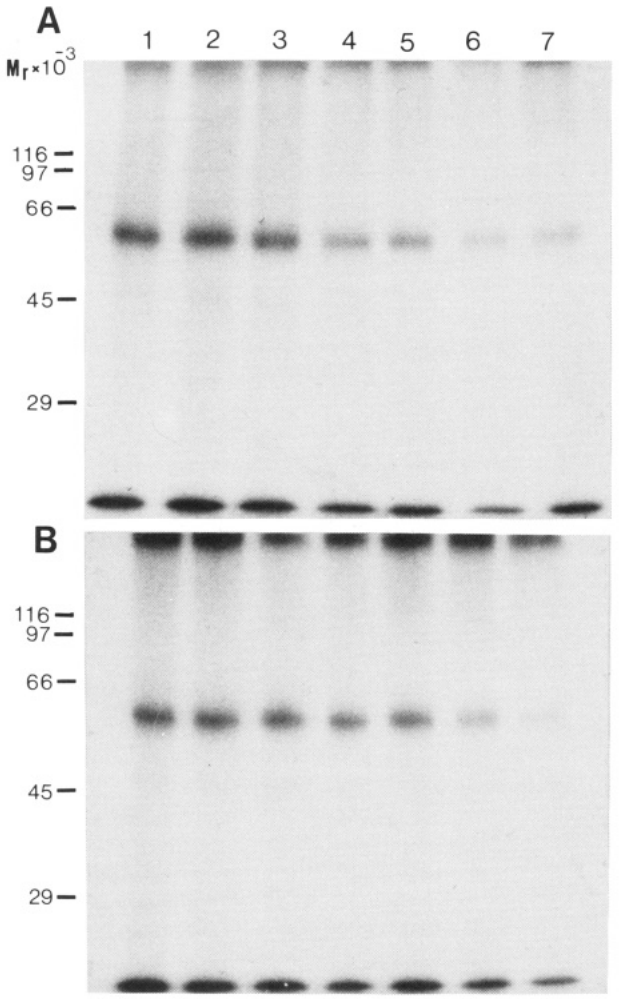

FIGURE 7: Competitive displacement of ^{125}I-LVP by increasing concentrations of unlabeled LVP (panel A) or V_1/V_2-antagonist (panel B). Lanes: (1) none (2) 50 nM, (3) 100 nM, (4) 500 nM, (5) 1 μM, (6) 5 μM, (7) 10 μM LVP or V_1/V_2-antagonist.

V_1/V_2-antagonist, only slightly by oxytocin, and not at all by angiotensin II. These results are consistent with those of Maggi et al. (1987), who found that AVP, LVP, and the V_1-antagonist have similar affinities for the vasopressin binding site of pig genital tract whereas the affinity of oxytocin for the same site is 100 times lower.

The rat liver vasopressin receptor can be labeled by [8-lysine]vasopressin although the hormone with antidiuretic and vasopressor activities in the rat is [8-arginine]vasopressin. This rat liver vasopressin receptor showed similar characteristics to the pig liver receptor. The displacement of the label by the V_1-antagonist and only partially by the V_2-agonist conclusively shows that the protein of M_r 60000 is the V_1 type of vasopressin receptor.

The concentration of ^{125}I-LVP at which incubation and cross-linking were performed excludes the possibility of binding at sites of low affinity. Therefore, the protein of M_r 60000 represents the presence of high-affinity binding sites. The K_d of LVP for the V_2 type of receptor from pig has been reported to be between 1 and 20 nM (Fahrenholz et al., 1985, 1988; Jard 1983b; Maggi et al., 1986) and for the V_1 has been found to be around 1 nM (Maggi et al., 1987).

The protein of a M_r 58000 revealed by the ligand affinity blotting might correspond with the band of M_r 60000 of the cross-linking experiments. The difference in the estimation of the molecular mass of the vasopressin receptor is probably due to the increase given by the LVP in the cross-linking experiments. A similar molecular form was labeled by using ^{125}I-AVP in porcine kidney, which expresses the V_2 type receptor (Aiyar et al., 1989). Similarly, Fahrenholz et al. (1988) found a major form of M_r 60000 in pig kidney using a photoactivable vasopressin analogue. The estimated M_r 60000 might be also comparable with the finding of Marie and Roy (1988), who were able to cross-link ^{3}H-LVP to a protein of M_r 50000 in membranes from the pig cell line LLC-PK$_1$. However, in the rat liver and ox kidney proteins of M_r 30000 and 38000 have been found as major forms (Boer & Fahrenholz et al., 1985). This led Fahrenholz et al. (1985) to postulate that the vasopressin receptor is a heterodimeric protein. Our results clearly show that the V_1 vasopressin receptor in the rat and pig livers is a protein of M_r 60000 and do not support the possibility of a dimeric structure since no change in the electrophoretic mobility was observed in the absence of reductant. Moreover, this finding also indicates that the receptor does not contain internal disulfide bonds that compact its structure as observed in other membrane receptors (Massagne et al., 1981; Moxham & Malbon, 1985). The difference between our results and those of Fahrenholz and co-workers (Boer & Fahrenholz, 1985; Fahrenholz et al., 1985) related to the assessment of the molecular mass might arise from proteolytic degradation of the receptor or, alternatively, from the different methods used to label the receptor. Indeed, in the photoaffinity labeling the hormone is modified by the introduction of the photoreactive label prior to binding and thus might change the affinity of the hormone for proteins whereas, in the cross-linking experiments, the cross-linking reaction is carried out after the iodinated hormone is bound to the receptor under equilibrium conditions.

A small difference in molecular mass seems to exist between the pig liver vasopressin receptor, that is, the [8-lysine]vasopressin receptor and the rat liver receptor or [8-arginine]vasopressin receptor. Further molecular characterization will be necessary to determine the degree of homology between these two receptors.

The molecular size of the vasopressin receptor determined by hydrodynamic methods and by radiation inactivation experiments is higher than the value of M_r 60000. It is difficult, however, to compare the molecular mass obtained by electrophoresis under reducing and denaturing conditions and that obtained by hydrodynamic or radiation inactivation methods since these last two methods determine the functional size of the macromolecules rather than that of the individual protein (Kempner & Schlegel 1979). It is known that the vasopressin receptor is associated with G proteins even when the receptor has been solubilized (Fitzgerald et al., 1986).

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Registry No. LVP, 50-57-7; AVP, 113-79-1; EGS, 62229-50-9; HSAB, 135190-30-6.

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