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Solubilization and Reconstitution of Vasopressin V_1 Receptors of Rat Liver

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SUMMARY

The observation that vasopressin receptors loose their binding capacity on solubilization suggests that lipid-milieu may be a requirement for the binding of vasopressin to these receptors. To preserve the ligand binding capacity, we have developed reconstitution procedures to study solubilized rat liver vasopressin receptors. We report here that vasopressin V_1 receptors were efficiently solubilized from rat liver membranes with egg lysolecithin and quantitatively reconstituted into phospholipid vesicles resulting in a 4-fold purification. Binding of [^{3}H]arginine vasopressin to the reconstituted receptors was saturable and of high affinity (K_d = 0.6 ± 0.1 nM). In competition binding experiments the solubilized, reconstituted receptors exhibited the same pharmacological profile as was observed with vasopressin V_1 receptors in membranes.

Vasopressin induces contraction of vascular smooth muscle and enhances water permeability of the renal collecting duct epithelium by activating V_1 and V_2 receptors, respectively (1). These receptor subtypes activate different second messengers and can be distinguished pharmacologically. V_1 receptors mediate stimulation of phosphatidylinositol turnover (2, 3) and inhibition of cAMP and cGMP accumulation induced by β-adrenergic agonists and atrial natriuretic factor, respectively (4, 5). V_2 receptors mediate stimulation of adenylate cyclase activity. AVP-binding sites have been characterized in rat liver using both hepatocyte and plasma membranes (6–8). To determine the molecular properties of the vasopressin receptors, it is necessary, as a first step toward purification, to extract the receptors from cell membranes in an active form. Previously, vasopressin receptors of liver and kidney membranes were solubilized as a hormone-receptor complex by non-ionic detergents (9). Solubilization of the receptors in the nonliganded state abolished hormone recognition (10, 11). Here we report that vasopressin V_1 receptors can be solubilized efficiently from rat liver membranes with egg lysolecithin; that the solubilized receptors can be quantitatively reconstituted in phospholipid vesicles; and that the reconstituted receptors retained the high affinity for AVP and V_1 binding characteristics similar to those observed in plasma membranes.

Materials and Methods

AVP and dDAVP were purchased from Bachem (Torrance, CA). [^{3}H]AVP (64.3 Ci/mmol) was from New England Nuclear (Boston, MA). Vasopressin analogs [d(CH_2)$_5$D-Tyr(Et)VAVP and d(CH_2)$_5$D-IleVAVP] were synthesized at SK&F Laboratories (Philadelphia, PA). Angiotensin II, bradykinin, and lysolecithin (L-α-lysophosphatidyl choline from egg yolk-type I) were from Sigma Chemical Co. (St. Louis, MO). Soybean phospholipid (asolectin) was from Associated Concentrates (Woodside, NY). PD-10 (prepacked disposable columns) was from Pharmacia Fine Chemicals, (Uppsala, Sweden).

Membrane preparation. Male Sprague-Dawley rats (225–275 g) were decapitated, and livers were removed rapidly and placed in ice-cold saline. The livers were minced and homogenized (100 g of liver per 200 ml of 1 mM $NaHCO_3$) in a Potter-Elvehjem homogenizer. The volume was adjusted to 2 liters with 1 mM $NaHCO_3$. The crude homogenate was filtered through two layers and subsequently four layers of cheesecloth. The filtrate was centrifuged at 2,000 × *g* for 10 min at 4°. The pellet was resuspended in ice-cold 0.25 M sucrose, 20 mM Hepes, pH 7.4, 2 mM EGTA (buffer A) for 15 min at 8,000 × *g*. The supernatant was centrifuged at 40,000 × *g* for 30 min at 4°. The membrane pellet was resuspended in buffer A at 5–7 mg of protein/ml and stored in liquid nitrogen. [^{3}H]AVP binding to rat liver membranes was determined for 60 min at 37° according to the method of Stassen *et al.* (12).

Solubilization procedure. Membrane fractions from rat liver in buffer A were homogenized with 0.4% ice-cold egg lysolecithin in buffer A. The mixture was incubated for 30 min at 0° and then centrifuged at 200,000 × *g* for 60 min at 4°. The supernatant was separated from the pellet and kept at 0° before the reconstitution.

Reconstitution of the solubilized proteins into phospholipid vesicles. Soybean phospholipid suspension was prepared by suspending the phospholipid up to 0.5% in 30 mM Hepes, pH 7.4, and 5 mM $MgCl_2$ (buffer B). The suspension was sonicated in a Branson bath

ABBREVIATIONS: AVP, 8-arginine vasopressin; dDAVP, 1-deamino, [8-D-arginine]vasopressin; d(CH_2)$_5$D-Tyr(Et)VAVP, 1-β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-(*O*-ethyl)tyrosine 4-valine]8-arginine vasopressin; d(CH_2)$_5$D-IleVAVP, 1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-D-isoleucine, 4-valine]8-arginine vasopressin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

TABLE 1

Solubilization and reconstitution of V_1 receptors of rat liver membranes

Binding of [^{3}H]AVP to rat liver membranes was done as described by Stassen *et al.* (12). The apparent dissociation constand (K_d) and specific activity were determined from the Scatchard analysis by an unweighted linear least squares fit. For the reconstituted fraction, the values were calculated from the data in Fig. 2.

Parameters	Membranes	Solubilized, reconstituted protein
Total protein (mg)	20.6 ± 2.8	4.24 ± 0.7
Specific activity (fmol/mg protein)	366 ± 16	1441 ± 52
Total activity (pmol)	7.5 ± 0.6	6.1 ± 0.4
K_d (nM)	0.8 ± 0.1	0.6 ± 0.1
Yield (%)	100	81

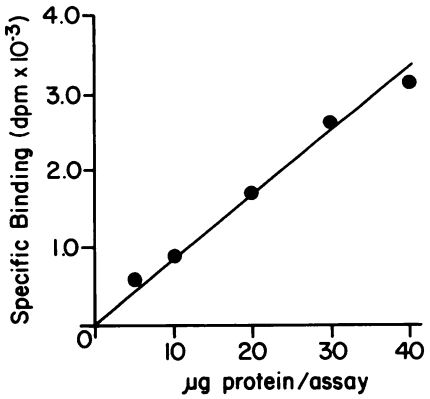

Fig. 1. Specific [^{3}H]AVP binding to reconstituted vasopressin receptors as a function of protein concentration. Binding was performed for 60 min at 37° with 1 nM [^{3}H]AVP and increasing concentrations of protein. Specific binding was defined as the difference between the total binding and the binding in the presence of 5 µM unlabeled AVP. Mean values of triplicate determinations are given. The errors were smaller than the *symbols*. The experiment was repeated with similar results.

sonicator for 10 min at 0° and centrifuged at 40,000 × *g* for 20 min. Egg lysolecithin was added to the supernatant up to 0.4%. The solubilized rat liver membrane fraction was mixed with an equal volume of phospholipid suspension supernatant containing egg lysolecithin and incubated at 0° for 15 min. The mixture was chromatographed in buffer B on a 2 × 40 cm column of Sephadex G-50. The void volume yielded a turbid fraction that contained 90% of the solubilized protein.

[^{3}H]AVP binding to solubilized reconstituted fraction. Binding was done at 37° for the indicated time in an incubation mixture (total volume of 250 µl) containing buffer B, solubilized reconstituted membrane fractions, and [^{3}H]AVP, with (nonspecific binding) or without (total binding) 5 µM AVP. Bound and free ligand were separated on PD-10 columns equilibrated with buffer B.

Protein determination. Protein was determined as described by Lowry *et al.* (13) and bovine serum albumin was the standard. The protein for the solubilized reconstituted fraction was given after subtracting the absorption for the identical fraction prepared in the absence of membrane protein.

Results and Discussion

[^{3}H]AVP binding to rat liver membranes yielded a K_d of 0.8 ± 0.1 nM and B_{max} of 366 ± 16 fmol/mg of protein. When the receptors were solubilized with egg lysolecithin (0.4%), less than 10% of the receptors were recovered in the pellet. The supernatant contained about 20% of the membrane protein. Specific binding of [^{3}H]AVP to the solubilized receptors was poor. Specific binding was markedly enhanced after transfer of the solubilized receptors to phospholipid vesicles. The incorporation of solubilized vasopressin receptors into phospholipid vesicles was achieved by mixing the solubilized rat liver membrane extract with soybean phospholipid. Chromatography of the mixture on Sephadex G-50 yielded all receptors in the void volume. Specific binding of 1 nM [^{3}H]AVP to the reconstituted receptors was 85%. Phospholipid vesicles without solubilized liver membranes displayed no specific [^{3}H]AVP binding. Approximately 80% of the membrane receptors of the preparation were recovered in the phospholipid vesicles (Table 1). The specific activity of the receptors was increased by about 4-fold. [^{3}H]AVP binding to the solubilized, reconstituted fraction did not change after (a) recentrifugation of the lysolecithin extract

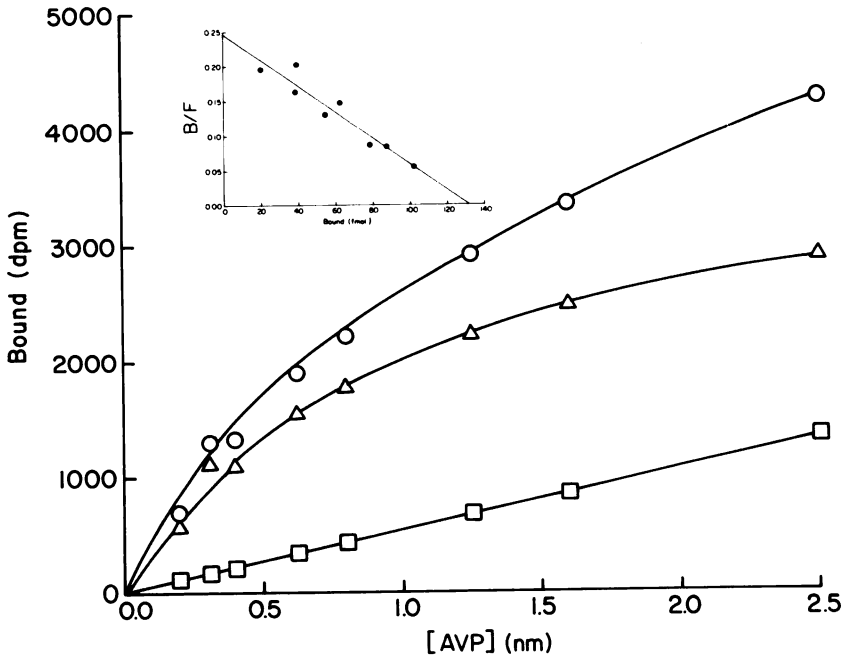

Fig. 2. Saturation equilibrium binding of [^{3}H]AVP to reconstituted vasopressin receptors. Binding was performed for 60 min at 37° with 50 µg of reconstituted protein and increasing concentrations of [^{3}H]AVP as described in the legend of Fig. 1. ○, total binding; □, nonspecific binding in the presence of 5 µM AVP; △, specific binding. *Inset*, Scatchard plot, K_d = 0.6 ± 0.1 nM, B_{max} 1441 ± 52 fmol/mg of protein. The data points are means of triplicate determinations. This experiment was representative of four similar experiments.

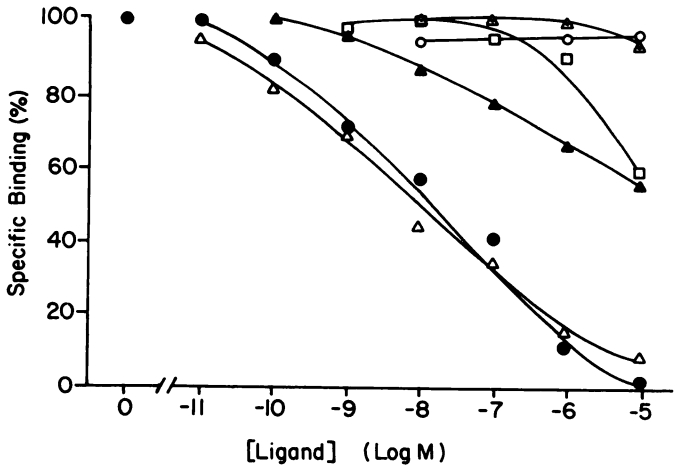

Fig. 3. Inhibition of specific [^{3}H]AVP binding to reconstituted vasopressin receptors. Binding was performed for 60 min with 1 nM [^{3}H]AVP, 50 μg of reconstituted protein, and unlabeled ligands at the indicated concentrations as described in the legend of Fig. 1. The data are expressed as per cent control of specific binding. △, AVP; ●, d(CH$_2$)$_5$D-Tyr(Et)VAVP; ▲, d(CH$_2$)$_5$D-IleVAVP; □, dDAVP; △, angiotensin II; ○, bradykinin. Each curve was replicated in at least two more experiments with each being performed in triplicate.

at 200,000 × g for 60 min or (b) filtration of the extract through Millipore 220-nm filters. In addition, electron microscopic examination of the lysolecithin extract confirmed the absence of any particulate matter, indicating that the receptors were solubilized.

The specific binding of the reconstituted receptors was abolished by incubating for 3 min at 100°. The reconstituted receptors were also destroyed by trypsin (5 μg of trypsin/ml of buffer B for 10 min at 37°). At 0.5 nM [^{3}H]AVP, specific binding to reconstituted vasopressin receptors was rapid and reached equilibrium after 30 min at 37°. Specific binding increased linearly with the protein concentration from 5 to 40 μg (Fig. 1). A typical saturation curve for [^{3}H]AVP binding to the solubilized reconstituted receptors is shown in Fig. 2. Scatchard analysis indicates a single population of binding sites with dissociation constant (K_d) of 0.6 ± 0.1 nM. The K_d value is similar to that obtained with receptors present on plasma membranes (Table 1).

The affinity of selective vasopressin agonists and antagonists was determined in competition binding studies (Fig. 3). The IC$_{50}$ values, which were calculated according to the method of Cheng and Prusoff (14) for inhibition of 1 nM [^{3}H]AVP by unlabeled AVP, d(CH$_2$)$_5$D-Tyr(Et)VAVP (a V$_1$/V$_2$ antagonist) (15), d(CH$_2$)$_5$D-IleVAVP (a V$_2$ antagonist) (16), and dDAVP (a V$_2$ agonist) were 3.0, 3.4, 56.0, and 1500 nM, respectively. Similar values have been reported for rat liver membranes by Stassen *et al.* (12). No inhibition was observed with angiotensin II or bradykinin. These data indicate that the AVP-binding sites that were solubilized and reconstituted retained a greater affinity for V$_1$ antagonist than for V$_2$ antagonist. Our preliminary experiments using CHAPS (5 mM), another commonly used zwitterionic detergent, suggests that a much higher [^{3}H]AVP concentration is needed to obtain saturation, and, furthermore, the antagonist competition of [^{3}H]AVP binding was poor (data not shown).

The data presented here indicate that rat liver V$_1$ receptors can be efficiently solubilized from rat liver membranes with egg lysolecithin and, most importantly, the solubilized receptors were shown by reconstitution in phospholipid vesicles to retain the appropriate ligand binding characteristics. Thus, using these methods, purification and further characterization of vasopressin V$_1$ receptors should now be possible.

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