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Covalent Labeling of Vasopressin Receptors from LLC-PK$_1$ Cells by the Use of a Bifunctional Reagent

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SUMMARY

The possibility of covalently attaching vasopressin to its receptors by the use of a bifunctional reagent was explored. Plasma membranes from the LLC-PK$_1$ pig kidney cell line were purified by Percoll density gradient centrifugation. These membranes contained a single population of high affinity (K_d = 5.2 nM) and high capacity (B_{max} 3.8 pmol/mg of protein) [^{3}H]lysine vasopressin ([^{3}H]LVP)-binding sites. [^{3}H]LVP-labeled receptors could be solubilized with a high yield (83%) and minimal dissociation (9%) by treatment with the non-ionic detergent, octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) (0.5%, v/v) in the presence of glycerol (20%). The solubilized [^{3}H]LVP-labeled receptors were stable upon storage at 4° (5% dissociation after 24 hr). They were partially purified to a specific activity of 17 pmol/mg of protein by chromatography on a Cibacron blue-Sepharose column with a yield of 90%. The [^{3}H]LVP-receptor complexes in both intact membranes and the partially purified preparation were almost completely dissociated by incubation at 30° for 30 min in the presence of 20 mM ethylenediaminetetraacetate (EDTA). This property was used to test the effect of ethylene glycol bis (succinimidyl-succinate) (EGS) as cross-linking reagent for the covalent attachment of [^{3}H]LVP to its receptors. After treatment of [^{3}H]LVP-labeled membranes for 30 min with 1 mM EGS at 4°, about 30% of specifically bound [^{3}H]LVP was resistant to EDTA dissociation. The amount of EDTA-resistant binding varied as a linear function of the fractional receptor occupancy and maximal binding capacity of the different batches of membranes used. Similar results were obtained with solubilized and partially purified vasopressin receptors. Upon steric exclusion high performance liquid chromatography, the EDTA-resistant [^{3}H]LVP-labeled material, like the native [^{3}H]LVP-labeled receptor, was eluted as a single and apparently homogeneous peak. The covalent character of the EGS-induced [^{3}H]LVP binding to solubilized or partially purified receptors was assessed by its resistance to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The yield of EGS-induced labeling deduced from these experiments (27%) was close to that determined by the EDTA method. SDS-PAGE analysis of the [^{3}H]LVP-labeled cross-linked material revealed the specific labeling of a major 50-kDa component and a minor component of 30 kDa. The size of these two components was not affected by dithiothreitol.

Vasopressin receptors from various tissues have been characterized (for review, see Ref. 1). On a functional basis, two vasopressin receptor subtypes were distinguished and were named V_2 and V_1 receptors (2). V_2 receptors mediate the antidiuretic response to vasopressin and are functionally coupled to adenylate cyclase (3). V_1 receptors are not coupled to adenylate cyclase, and current experimental evidence indicates that calcium might be the second messenger of vasopressin acting through V_1 receptors (reviewed in Ref. 1). It was recently shown that V_1 receptors are coupled in a GTP-dependent manner to a phospholipase C activity catalyzing the formation of inositol 1,4,5-triphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphosphate (4, 5).

Studies on the structure and physicochemical properties of V_1 and V_2 vasopressin receptors have been hampered by the lack of appropriate probes. Recently, Fahrenholz and colleagues have designed photoactivable vasopressin analogues allowing covalent labeling of V_2 vasopressin receptors from rat and bovine kidney (6) and V_1 receptors from rat liver (7, 8). However, as is frequently observed with photoactivable ligands, the yield of covalent labeling of vasopressin receptors was low (about 2%). In the present study, we explored the possibility that cross-linking with a cleavable bifunctional cross-linking reagent might lead to a higher yield of receptor labeling. This method has been successfully used for labeling angiotensin (9), prolactin (10), follitropin receptors (11), and vasoactive intestinal polypeptide receptors (12).

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ABBREVIATIONS: EGS, ethylene glycol bis (succinimidyl-succinate); [^{3}H]LVP, ([^{3}H]$_2$Tyr^2) lysine vasopressin; LVP, lysine vasopressin, HSA, human serum albumin; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; $C_{12}E_9$, nonaethylene glycol mono-*n*-dodecyl ether; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; EDTA, ethylenediaminetetraacetate; Hepes, 4-(2-hydroxyethyl ether)-1-piperazineethanesulfonic acid.

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The experiments were carried out on LLC-PK_1 cells (pig kidney cell line) which can be produced in large amounts and express on their surface a large number of V_2-type vasopressin receptors (150,000 sites/cell) (13). For these reasons LLC-PK_1 cells represent a convenient biological material for studies on vasopressin receptors. A possible disadvantage of using cross-linking reagents to covalently attach vasopressin to its receptor is the cross-linking of the receptor with other membrane components (14). To circumvent these difficulties, much effort was devoted to the design of experimental procedures allowing: 1) a high yield solubilization of reversible vasopressin-receptor complexes formed on the membrane, 2) a partial purification of these complexes, and 3) a specific cross-linking of vasopressin to its receptors.

We report that the cross-linking reagent EGS (15) allowed covalent labeling of vasopressin receptors from LLC-PK_1 cells with a high yield (30%). Physicochemical properties of the covalently labeled material as deduced from HPLC steric exclusion and SDS-PAGE are also reported.

Materials and Methods

Products. [^{3}H]LVP (17 Ci/mmol) was prepared as already described (16) and purified by affinity chromatography using a neurophysin-Sepharose column (17). The radiochemical purity of the peptide was controlled by HPLC using a μBondapak C_{18} column from Waters. Other products used were purchased from the following sources: LVP from Bachem (Buddendorf, Switzerland), Triton X-100 from Koch-Light Laboratories, CHAPS from Serva (Tebu S.A., Le Peray en Yvelines, France), $C_{12}E_8$ from Hoechst (Stuttgart, FRG), standards for SDS-PAGE and steric exclusion chromatography Percoll, Sepharose CL6B and Cibacron blue-Sepharose CL4B from Pharmacia Fine Chemicals (Uppsala, Sweden), Bio-Gel P-30 from Bio-Rad Laboratories (Richmond, CA), $C_{12}E_9$ and HSA from Sigma (Saint Louis, MO), and EGS and Cibacron blue F3GA from Pierce Chemical Co (Rockford, IL). Cibacron blue F3GA contains reactive chlorine present on the triazine group. It was desactivated before use by treatment with 0.1 N NaOH for 16 hr at 60° (18). Other chemicals were of the highest purity available.

Cell culture. Pig kidney cells from the LLC-PK_1 (ATCC CL101) and LLC-PK_1L lines were used. LLC-PK_1 cells were grown to confluency in glass or plastic Petri dishes (150 cm^2) using Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 20 units/ml penicillin, and 20 μg/ml streptomycin. Subculturing was performed as described in Ref. 19. LLC-PK_1L cells (a generous gift of Dr. J. S. Handler, National Institutes of Health, Bethesda, MD) were grown in a chemically defined medium (Coon's modified Ham's F12 medium to which was added 10 mM selenium, 50 units/ml penicillin, and 50 μg/ml streptomycin. LLC-PK_1L cells with different densities of vasopressin receptors were prepared by growing the cells in minimal medium complemented with hormones and vitamins as described (20) or by growing the cells in the presence of butyrate (2.5 mM) added to the minimal or enriched medium.

Membrane preparation. Membranes were prepared from LLC-PK_1 cells as follows. One week after seeding, the dishes were washed three times with 20 ml of ice-cold phosphate-buffered serum solution without Ca^{2+} and Mg^{2+}. Cells were scraped with a rubber policeman, suspended in 6 ml of a solution composed of 50 mM Tris-HCl, pH 7.4, and 1 mM EDTA. They were homogenized with a tight-fitting Dounce homogenizer (15 strokes). The homogenate was centrifuged at 50 × g for 10 min to eliminate large cellular debris. The supernatant was centrifuged at 12,000 × g for 10 min. The pellet was resuspended in 5.5 ml of a solution (gradient buffer) composed of 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 200 mM sucrose, and 1 mM EDTA and dispersed with a Teflon Elvehjem-Potter homogenizer. The 10,000 × g pellets corresponding to 10 mg of protein were mixed with a Percoll solution (30%, v/v, relative to the suspension supplied by Pharmacia Fine Chemicals) made in gradient buffer (33 ml/tube). Density gradient was formed by centrifugation at 22,000 rpm for 30 min in a Beckman 50-2 Ti rotor. The gradient was fractionated by pumping at the bottom of the tube. The first lowest 23 ml were discarded and the next 5.5 ml containing the bulk of membranes were collected. Percoll was eliminated by centrifugation at 45,000 rpm for 90 min in a Beckman 50-2 Ti rotor. The pellet was dispersed in the gradient buffer and the membrane preparation (1.5 mg of protein/ml) was stored in liquid nitrogen.

[^{3}H]LVP binding assay on membranes. Membranes (0.5 mg/ml) were incubated for 20 min at 30° in 100 μl of an incubation medium containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 200 mM sucrose, and various amounts of [^{3}H]LVP. Total binding was corrected for nonspecific binding measured in the presence of 2 μM unlabeled LVP. Bound and free [^{3}H]LVP were separated by filtration on Gelman filters (GA3, 1.2 μm) as previously described (21).

Solubilization of hormone-receptor complexes. Membranes (0.5 mg/ml) were incubated in the presence of 10 nM [^{3}H]LVP as described above. After cooling at 4°, free [^{3}H]LVP was eliminated by centrifugation at 45,000 × g for 30 min. The membrane pellet was homogenized in 50 mM glycylglycine/NaOH (or, when indicated, Hepes), pH 8.6, 5 mM $MgCl_2$, 200 mM sucrose, 1 mM phenylmethanesulfonyl fluoride, and different concentrations of the detergents tested. When indicated, NaCl and glycerol were also present. The mixture was incubated for 90 min at 0° and sedimented at 222,000 × g at 4° for 60 min. The supernatant designated as the soluble fraction was collected. Separation of free from bound radioactivity in the soluble fraction was achieved as previously described (20) by filtration through Bio-gel P-30 (100–200 mesh) columns (0.7 × 6.5 cm) equilibrated with a solution composed of 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 200 mM sucrose, 100 mM NaCl, 0.02% (w/v) NaN_3, and 0.1% (v/v) Triton X-100.

Partial purification of vasopressin receptors. A Cibacron blue-Sepharose CL6B column (2.5 × 3 cm) was equilibrated at 4° with a Hepes buffer [50 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 200 mM sucrose, 0.1 mM phenylmethanesulfonyl fluoride, and 0.05% (v/v) $C_{12}E_8$]. A soluble fraction of [^{3}H]LVP-labeled membranes (120 mg of protein in 300 ml) was applied to the column at a 125-ml/min flow rate. The column was washed with 150 ml of Hepes buffer, and 25 ml of a desactivated Cibacron blue F3GA solution (1 mg/ml) were applied to the column. The column was then washed with 100 ml of Hepes buffer and 5-ml fractions were collected. Bound radioactivity present in these fractions was measured by filtration on Bio-Gel columns (see above). Fractions containing bound radioactivity were pooled. Cibacron blue in the pooled fractions was eliminated by adsorption on an HSA-Sepharose column. HSA was coupled to Sepharose CL-4B with the cyanogen bromid method. The HSA-Sepharose gel (10–12 mg of HSA/g of Sepharose gel) was packed in a column (2.5 × 10 cm) and was equilibrated at 4° in Hepes buffer. The pooled fractions from the Cibacron blue-Sepharose column were applied at a flow rate of 1 ml/min to the HSA-Sepharose column, and the column was washed with Hepes buffer. The partially purified [^{3}H]LVP-labeled material was recovered in a total volume of 100 ml.

Cross-linking of [^{3}H]LVP bound to vasopressin receptors. The effect of EGS as a cross-linking agent for [^{3}H]LVP bound to its receptor was tested both on intact membranes and on solubilized and partially purified vasopressin receptors.

For experiments with intact membranes, LLC-PK_1 or LLC-PK_1L cell membranes (0.5 mg/ml) were incubated with different concentrations of [^{3}H]LVP as described above. In order to eliminate the Tris buffer and the bulk of free [^{3}H]LVP, the membrane suspension was centrifuged at 45,000 × g at 4° for 30 min and the pellet was resuspended at the same concentration in a solution composed of 50 mM Hepes, pH 8.0, 5 mM $MgCl_2$, and 200 mM sucrose. Membranes were incubated in the presence of various concentrations of EGS for 45 min at 4° and under constant shaking. EGS was dissolved in DMSO immediately before use and its concentration was adjusted so that the final concentration of DMSO during the incubation of membranes was constant in

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all experiments (0.5%, v/v). At the end of the incubation in the presence of EGS, lysine (50 mM, final concentration) was added to quench the EGS effect. Aliquots incubated with lysine (50 mM) at 4° for 5 min before the addition of EGS were used as controls to test the efficacy of lysine as a quenching agent and the influence of DMSO. Experiments with membranes which were incubated in the presence of [^{3}H]LVP and a 100-fold excess of unlabeled LVP were run in parallel.

Cross-linking experiments on solubilized receptors were conducted as follows. Soluble extracts (in Hepes buffer) or partially purified receptors were incubated in the presence of EGS under constant shaking at 4° for 100 min. EGS (1 mM final concentration) was added to the incubation medium at 20-min intervals from the beginning of the incubation. An aliquot was collected before each EGS addition. The reaction was stopped by adding 50 mM lysine. Control experiments with soluble extracts prepared from membranes incubated in the presence of [^{3}H]LVP plus a 100-fold excess of unlabeled LVP were run in parallel. In some experiments [^{3}H]LVP bound to solubilized receptors was dissociated before EGS treatment.

For both the experiments with intact membranes and solubilized receptors, the effect of EGS was estimated by measuring the amount of bound radioactivity which was resistant to incubation in the presence of EDTA (20 mM) for 30 min at 30°. Bound radioactivity was separated from free radioactivity by filtration through Gelman filters (membranes) or Bio-Gel P-30 columns (solubilized extracts).

For further analysis of the physicochemical properties of EDTA-resistant [^{3}H]LVP-labeled material, EGS-treated soluble extracts or partially purified receptors were dialyzed overnight against 10 mM Tris-HCl, pH 7.4, and 0.01% (v/v) $C_{12}E_8$ and lyophilized. Control experiments with freshly prepared samples indicated that lyophilization did not alter the properties of the EDTA-resistant [^{3}H]LVP-labeled material.

Steric exclusion HPLC of solubilized [^{3}H]LVP-labeled vasopressin receptors. Tandem-linked, 60-cm TSK 4000 and TSK 3000 steric exclusion columns from LKB (Bromma, Sweden) were used. Columns were equilibrated at 4° with a medium composed of 150 mM Na_2HPO_4, pH 7.2, 5 mM $MgCl_2$, 0.05% (v/v) $C_{12}E_8$, and, when indicated, 200 mM sucrose. Samples (100 μl) of EGS-treated soluble material were filtered through a 0.2-μm Millipore membrane, applied to the columns, and eluted with the equilibration buffer at a flow rate of 0.5 ml/min. Fractions of 0.5 ml or 0.6 ml were directly collected in counting vials. Protein standards used for the calibration of the column were thyroglobulin (669 kDa), aldolase (150 kDa), bovine serum albumin (69 kDa), ovalbumin (43 kDa), and chymotrypsinogen (25 kDa).

SDS-PAGE of solubilized [^{3}H]LVP-labeled vasopressin receptors treated with EGS. PAGE was performed on thick (0.25 cm) slab gels according to the method of Laemmli (22). A 5% stacking gel and a 12.5% separating gel were used. Appropriated aliquots of lyophilized soluble extracts were homogenized and incubated for 1 hr at 30° in 1.5 ml of 80 mM Tris-HCl, pH 6.0, 2% SDS, 10% glycerol, 0.006% (w/v) bromophenol blue, and, when indicated, 100 mM DTT. The samples were centrifuged for 5 min at 12,000 rpm in an Eppendorf 5414 centrifuge and the supernatants were applied to the entire width (10 cm) of the gel. After electrophoresis (50 V), gels were sliced, and the slices were (0.2 cm) dried, homogenized, placed in counting vials, and digested overnight. Digestion was ensured by incubating the slices at 40° in a solution composed of 95 parts of 30% (v/v) H_2O_2 and 5 parts of NH_4OH. Vials were cooled and 0.2 ml of acetic acid was added before mixing with 12 ml of aqueous counting scintillation medium (ACS II, Amersham). Under these conditions, pieces of gel were uncompletely digested. Nevertheless, the yield of recovery of the counted radioactivity in the whole gel was about 72%. This yield was taken into account to calculate the yield of the cross-linking reaction.

Protein molecular weight standards used were phosphorylase (94 kDa) serum albumin (67 kDa), ovalbumin (43 kDa), carboxyanhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa). Standard proteins were stained with 0.25% Coomassie blue R250 (Sigma) in 7% acetic acid, 50% methanol and were destained with 7% acetic acid, 7% methanol.

Protein assays. The methods of Lowry *et al.* (23) and Peterson (24) were used for the determination of the protein content of membranes and soluble extracts, respectively.

Results

Experiments with intact membranes. LLC-PK$_1$ cell membranes purified by the Percoll density gradient method had a maximal [^{3}H]LVP binding capacity of 3.83 ± 0.45 pmol/mg of protein (mean ± SD of seven determinations). The corresponding value measured in the 10,000 × *g* fraction of an LLC-PK$_1$ cell homogenate was 1.5 ± 0.1 pmol/mg of protein (mean ± SD of five determinations). For both the crude and partially purified membranes, determination of dose-dependent [^{3}H]LVP binding indicated that [^{3}H]LVP interacted with a single population of sites. The determined K_d values were 5.2 ± 1.4 (five determinations) and 2.9 ± 0.5 nM (seven determinations), respectively. With respect to the 10,000 × *g* pellet loaded in the gradient, the yield of vasopressin receptors present in the preparation used was 70–80%. Nonspecific [^{3}H]LVP binding to purified membranes represented 8% of total binding for a [^{3}H]LVP concentration of 10 nM.

Fig. 1 illustrates the extent of the amount of specific irreversible binding of [^{3}H]LVP as a function of EGS concentration. Without EGS treatment, [^{3}H]LVP specifically bound to LLC-PK$_1$ cell membranes could be almost completely released

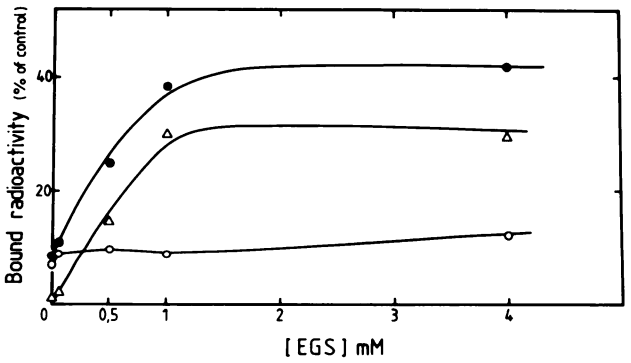

Fig. 1. Dissociation by EDTA of [^{3}H]LVP bound to LLC-PK$_1$ cell membranes: effect of EGS. Purified membranes (0.5 mg of protein) were incubated for 20 min at 30° with 5 nM [^{3}H]LVP in the presence or absence of 2 μM unlabeled LVP in a total volume of 1 ml. Bound radioactivity was determined by filtration (triplicate determinations on 50-μl aliquots of the membrane suspension) and counting. Total and nonspecific binding were 1.8 and 0.11 pmol/mg of protein, respectively. The value of specific binding was used as a reference to express all other values. Free [^{3}H]LVP in the membrane suspension was eliminated by centrifugation. Samples of the [^{3}H]LVP-labeled membranes were incubated in the presence of EGS at the indicated concentrations (●). Control samples (○) in which 50 mM lysine was added before EGS were run in parallel (for details see Materials and Methods). Finally, all samples were incubated in the presence of 20 mM EDTA for 30 min at 30° and the residual bound radioactivity was determined by filtration. All values are differences between the data derived from membranes incubated in the presence of [^{3}H]LVP alone and the corresponding data derived from membranes incubated in the presence of [^{3}H]LVP plus an excess of unlabeled LVP. They are expressed as a percentage of the [^{3}H]LVP specific binding determined before EGS treatment at the beginning of the experiment. △, differences between experimental (●) and control (○) data, representing the EGS-induced, EDTA-resistant [^{3}H]LVP binding. Each point represents the average of triplicate determinations performed in a single experiment. At 1 mM, the percentage of irreversibly bound radioactivity was 28 ± 4% (mean ± SD of four experiments made in triplicate).

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during the course of a 30-min incubation period at 30° in the presence of 20 mM EDTA. The dissociation was only partial when EDTA treatment was applied to [^{3}H]LVP-labeled membranes previously exposed to EGS for 30 min at 4°. With the highest EGS concentrations used (1–4 mM), about 30% of specific [^{3}H]LVP binding was resistant to EDTA-induced dissociation. In all further experiments EGS was used at a 1 mM concentration. It was checked that EGS was the active agent. Incubation of membranes in the presence of DMSO (0.5%), which was also present in the incubation medium (see Materials and Methods), did not alter the stability of [^{3}H]LVP-vasopressin receptor complexes. By contrast, treatment of unlabeled membranes with EGS prevented further specific [^{3}H]LVP binding. The presence of vasopressin bound to its receptors protected the membranes from this EGS-induced loss in binding capacity (data not shown).

The EDTA-resistant [^{3}H]LVP binding to EGS-treated membranes was proportional to the amount of [^{3}H]LVP which was bound before the addition of EGS and represented 28% of that amount (Fig. 2A). Similarly (Fig. 2B), for a given fractional receptor occupancy of 50%, the amount of EDTA-resistant [^{3}H]LVP binding was proportional to the maximal binding capacity of the membrane preparations used. To test that point, we took advantage of the possibility of modulating the vasopressin-binding capacity of LLC-PK$_1$L by modifying the composition of the chemically defined culture medium. Membranes were prepared from four batches of cells with different [^{3}H]LVP maximal binding capacities.

The EDTA-resistant, EGS-induced binding of [^{3}H]LVP binding to membrane was also resistant to membrane solubilization by SDS, suggesting that it did represent [^{3}H]LVP which had been covalently cross-linked to its receptors (or to other membrane components). However, preliminary experiments indicated that the covalently bound [^{3}H]LVP in solubilized membranes was associated with large aggregates (not shown). For this reason, all further attempts to covalently attach [^{3}H]LVP to its receptors were performed using solubilized and partially purified [^{3}H]LVP-labeled vasopressin receptors.

Solubilization and partial purification of [^{3}H]LVP-labeled vasopressin receptors. Among the non-ionic detergents tested, $C_{12}E_8$ and $C_{12}E_9$ were found to be the most efficient for solubilizing [^{3}H]LVP-labeled vasopressin receptors from LLC-PK$_1$ cell membranes with minor dissociating effect (Table 1). Under optimal conditions [0.5% (v/v) $C_{12}E_8$, 20% (v/v) glycerol] 83 ± 4% (mean ± SD of six determinations) of the [^{3}H]LVP-labeled vasopressin receptors were solubilized with minor dissociation (9%). It should be noticed that, after solubilization, glycerol could be eliminated and the concentration of $C_{12}E_8$ reduced to 0.05% (v/v) without affecting the stability and solubility of [^{3}H]LVP-labeled vasopressin receptors. Under the same conditions, only 43% of the membrane proteins were solubilized, leading to an increase of the specific activity of the [^{3}H]LVP-labeled vasopressin receptors from 3.1 + 0.2 pmol/mg of protein in the membrane fractions to 6.4 ± 0.7 pmol/mg of protein (mean ± SD of six determinations) in the soluble extract. By comparison, at optimal concentrations, the zwitterionic detergent CHAPS (0.5%, w/v) or the non-ionic detergent Triton X-100 (0.1%, v/v) solubilized a small proportion of the [^{3}H]LVP-labeled vasopressin receptors (13% and 20%, respectively). Glycerol (20%, v/v) increased the yield of solubilization by Triton X-100 but in the same time increased the dissociation of bound hormone. Glycerol had no effect on solubilization by CHAPS. Under all conditions indicated in Table 1, the yield of solubilization of total membranes proteins was 60–70% except for $C_{12}E_8$ + 20% glycerol (45%).

Partial purification of [^{3}H]LVP-labeled vasopressin receptors was achieved by affinity chromatography on Cibacron blue-Sepharose. As shown in Fig. 3, more than 90% of solubilized [^{3}H]LVP-labeled vasopressin receptors could be adsorbed on a Cibacron blue-Sepharose column. Minimal dissociation occurred during adsorption, washing of the column, and elution with desactivated Cibacron blue F3GA. The amount of undissociated [^{3}H]LVP-labeled vasopressin receptors recovered in the eluate represented 84 ± 2% (mean ± SD of six experiments) of the [^{3}H]LVP-labeled vasopressin receptors applied to the Cibacron blue-Sepharose column. The specific activity of the [^{3}H]LVP-labeled vasopressin receptors in the eluate was 17 ± 2 pmol/mg of protein (mean ± SD of six determinations), i.e.,

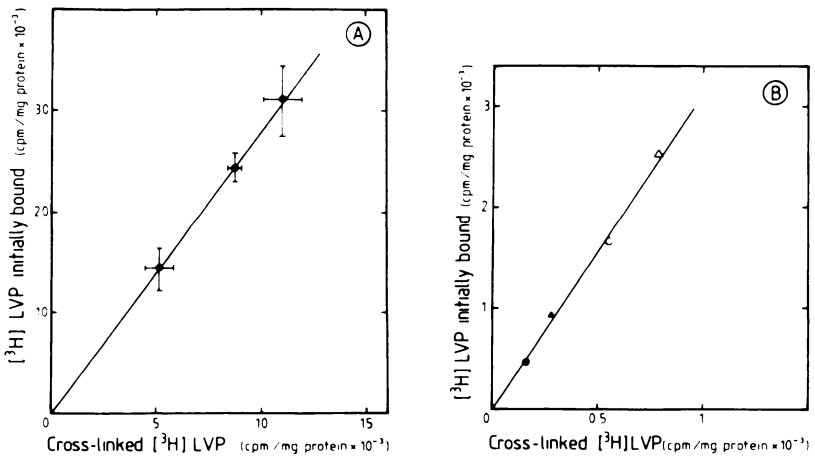

Fig. 2. EDTA-resistant vasopressin binding to LLC-PK$_1$ and LLC-PK$_1$L membranes after EGS treatment: relation to fractional receptor occupancy and maximal binding capacity. A. Membranes (0.5 mg of protein) were incubated in the presence of 2, 5, or 10 nM [^{3}H]LVP and the specific [^{3}H]LVP binding was determined. The membranes were treated with 1 mM EGS as indicated under Materials and Methods. The EDTA-resistant binding was determined as indicated in the legend to Fig. 1 and plotted as a function of the corresponding values of [^{3}H]LVP specific binding determined at the beginning of the experiment. Linear regression analysis gave an *r* value of 0.988. Each point represents the average of three experiments made in triplicate (mean ± SD). B. LLC-PK$_1$L cells were grown in minimal medium (●), enriched medium (▲), minimal medium complemented with 2.5 mM butyrate (○), or enriched medium complemented with 2.5 mM butyrate (△). Membranes were prepared from these cells and incubated with 5 nM [^{3}H]LVP. Specific [^{3}H]LVP binding was measured and the values obtained were plotted as a function of the corresponding values of EDTA-resistant binding determined after treatment with 1 mM EGS. Linear regression analysis gaven an *r* value of 0.996. Each point shows the average of quadruplicate determinations obtained from one experiment (variation < 5%).

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TABLE 1

Solubilization of [^{3}H]LVP-labeled vasopressin receptors from LLC-PK$_1$ cell membranes by different detergents

Purified membranes were incubated with 10 nM [^{3}H]LVP. Free [^{3}H]LVP was eliminated by centrifugation. The [^{3}H]LVP-labeled membranes (3 pmol/mg of protein) were resuspended at a concentration of 0.5 mg of protein/ml and incubated for 1 hr at 4° with the indicated detergents in the presence or absence of 20% (v/v) glycerol. The detergent-treated membranes were centrifuged at 220,000 × *g* for 1 hr at 4° and bound radioactivities in the supernatant (A) and the pellet (B) were determinated by filtration through Bio-Gel P-30 or Gelman filters, respectively. The data are expressed as a percentage of the amount of starting bound [^{3}H]LVP. This table summarizes the results of an experiment made with concentration of each detergent that gave the maximum solubilization with minor dissociation of [^{3}H]LVP-receptor complexes. The fractional dissociation of [^{3}H]LVP occurring during the solubilization procedure was calculated from the difference between total and bound radioactivities in the soluble fraction and is indicated in C. All values were corrected with nonspecific binding determined by incubating membranes with 2 μM unlabeled vasopressin.

Treatment		A	B	C
Detergent	Glycerol			
CHAPS 0.5% (w/v)	−	13	68	18
	+	13	71	16
Triton X-100 0.1% (v/v)	−	6	67	26
	+	20	42, 5	38
$C_{12}E_9$ 0.5% (v/v)	+	61	7	32
$C_{12}E_8$ 0.5% (v/v)	−	57, 5	31	12, 5
	+	83	8	9

3.5 times the specific activity in the crude solubilized material. It should be noticed that none of the agents frequently used to elute proteins adsorbed on Cibacron blue (NAD^+, high ionic strength, urea, ethylene glycol) (see, for instance, Ref. 25) were efficient to elute [^{3}H]LVP-labeled vasopressin receptors adsorbed on the column. Cibacron-blue F3GA present in the eluate was eliminated by passing the partially purified [^{3}H]LVP-labeled vasopressin receptors through an HSA-Sepharose column. This procedure permits dye-free, partially purified, [^{3}H]LVP-labeled vasopressin receptors to be obtained with a yield of 89 ± 2% (mean ± SD of six determinations) and with minimal dilution.

Effect of EGS on the stability of solubilized and partially purified [^{3}H]LVP-labeled vasopressin receptors. Treatment with EDTA (20 mM) for 30 min at 30° of solubilized and partially purified [^{3}H]LVP-labeled vasopressin receptors resulted in an almost complete (93%) release of bound radioactivity. When [^{3}H]LVP-labeled vasopressin receptors were treated with EGS (1 mM), about 20% of bound radioactivity was not released by subsequent EDTA treatment. This proportion of EDTA-resistant binding was increased to 40% by successive treatments with EGS (1 mM) (Fig. 4). Similar results were obtained with crude soluble extracts of [^{3}H]LVP-prelabeled membranes (not shown).

Analysis of cross-linked [^{3}H]LVP-labeled vasopressin receptors by size exclusion chromatography and SDS-polyacrylamide slab gel. [^{3}H]LVP bound to solubilized and partially purified vasopressin receptors was eluted from an HPLC steric exclusion column as a single and apparently homogeneous peak (Fig. 5A) with a retention volume comprise between those of thyroglobulin and aldolase. The EDTA-resistant fraction of EGS-treated [^{3}H]LVP-labeled vasopressin receptors had an almost superimposable elution profile (Fig. 5A). Similar results were obtained when using crude soluble extracts or partially purified [^{3}H]LVP-labeled vasopressin receptors. As shown in Fig. 5B, radioactivity associated with high molecular weight components was absent when the soluble

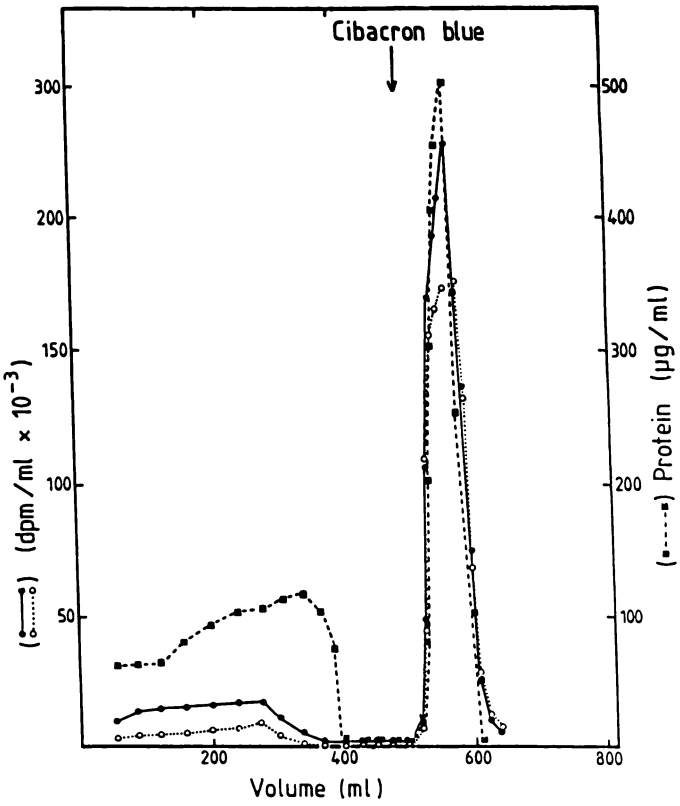

Fig. 3. Partial purification of solubilized [^{3}H]LVP-labeled vasopressin receptors by chromatography on a Cibacron blue F3GA-Sepharose column. Vasopressin receptors from LLC-PK$_1$ purified cell membranes (120 mg of protein) were labeled with 10 nM [^{3}H]LVP. Membranes were solubilized and the 220,000 × *g* supernatant of the solubilizate (250 ml) containing 9 × 10^6 dpm bound radioactivity was applied at a flow rate of 1.25 ml/min to a Cibacron blue-Sepharose column (2.5 × 3 cm) equilibrated with 0.05% (v/v) $C_{12}E_8$. The column was washed with 250 ml of Hepes buffer before elution with 25 ml of the Hepes buffer containing desactivated Cibacron blue F3GA (1 mg/ml). The column was washed with an additional 100-ml volume of Hepes buffer. For details, see Materials and Methods. Total (●) and bound (○) radioactivity values were determined in each fraction of the eluate as well total protein content (■).

extracts were treated with EDTA before addition of EGS. In this case only the radioactivity corresponding to the free hormone was recovered.

In the presence of DTT, polyacrylamide gel electrophoresis of EGS-treated, solubilized and partially purified [^{3}H]LVP-labeled vasopressin receptors (Fig. 6) revealed two peaks. A major peak had a molecular weight of 49,300 ± 6,000 (mean ± SD of five determinations) and a minor one with a molecular weight of 29,500 ± 400 (mean ± SD of five determinations). Radioactivity recovered under these two peaks represented 21.5 ± 1.7% and 5.7 ± 0.5% (means ± SD of five determinations) of the total radioactivity present in the gel, respectively, or 14.9 ± 3.5% and 3.8 ± 0.7% relative to the radioactivity applied to SDS-PAGE [the yield of recovery of total radioactivity in the gel was 71.8 ± 12.4% (mean ± SD of six determinations)]. Thus, about 27% of [^{3}H]LVP bound to EGS-treated solubilized and partially purified vasopressin receptors was resistant to SDS and electrophoresis under denaturing conditions, clearly suggesting the covalent character of the [^{3}H]LVP binding. No significant covalent binding of [^{3}H]LVP could be detected when the solubilized and partially purified [^{3}H]LVP-labeled vasopressin receptors were incubated in the presence of EDTA

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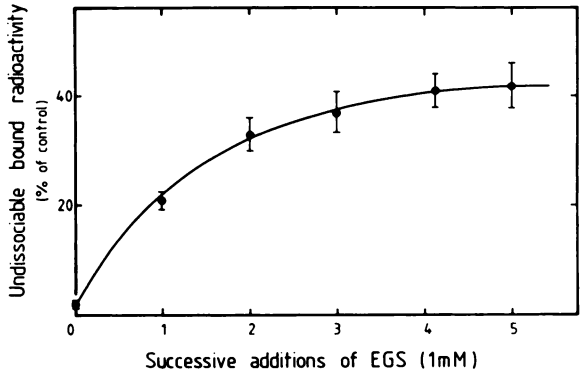

Fig. 4. EDTA-resistant vasopressin binding to solubilized and partially purified [^{3}H]LVP-labeled vasopressin receptors treated with EGS. Solubilized [^{3}H]LVP receptor complexes were purified on a Cibacron blue-Sepharose column as described under Materials and Methods. The Cibacron blue in the eluate was eliminated by chromatography on an HSA-Sepharose column. The partially purified [^{3}H]LVP-labeled vasopressin receptors (1.6×10^6 cpm in a total volume of 100 ml) was incubated for 100 min at 4° in the presence of EGS (1 mM final concentration) added at 20-min intervals from the beginning of the incubation. Before each EGS addition an aliquot of the incubation medium was collected and incubated in the presence of EDTA. The bound radioactivity (EDTA-resistant binding) was then determined. Values are expressed as a percentage of the control value determined before EGS treatment (10^5 cpm bound/mg of protein). Values on the graph are means ± SD of five independent experiments.

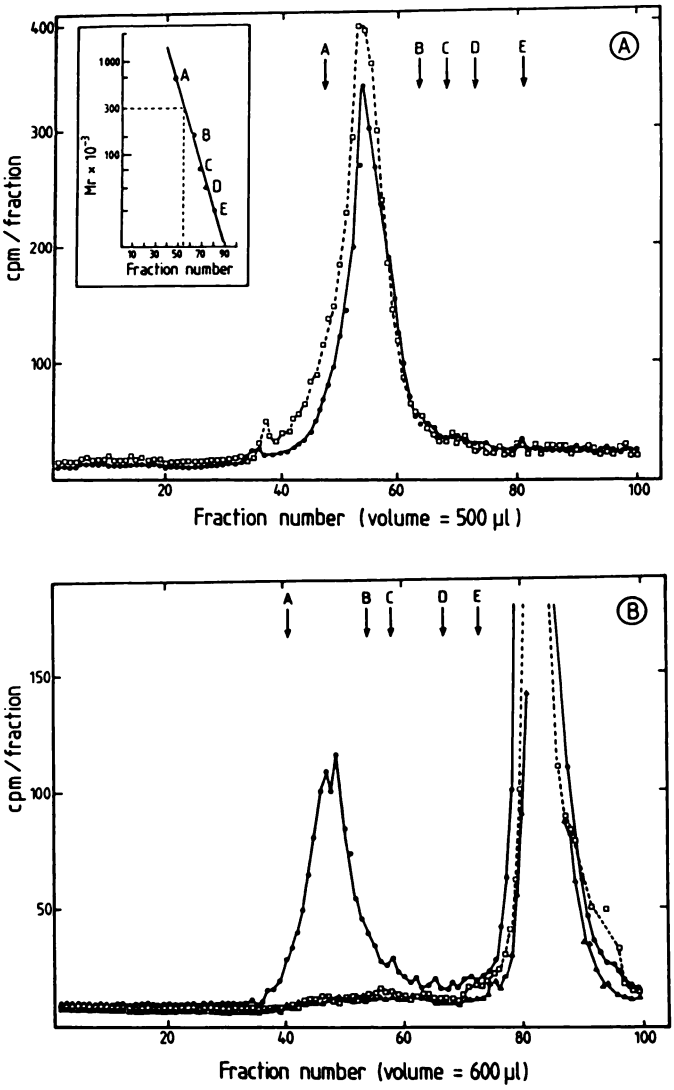

Fig. 5. Steric exclusion HPLC of the solubilized and partially purified [^{3}H]LVP-labeled vasopressin receptors: influence of EGS. [^{3}H]LVP-labeled vasopressin receptors were prepared in their solubilized and partially purified form. Three samples were treated with EGS, one of which was incubated in the presence of EDTA before EGS treatment. Two other samples were not treated with EGS and served as controls. All samples were concentrated by filtration through an Amicon YM 30 membrane under nitrogen pressure. Aliquots (100 µl) of the concentrated samples were applied to tandem-linked TSK4000 and TSK 3000 columns. For details see Materials and Methods. A. The elution buffer contained 5 mM $MgCl_2$ and the elution was performed at 4°. The figure shows the elution profiles of bound radioactivity in an EGS-treated sample (●) and in a control (□) sample. In the *inset* the calibration curve of the column is shown: *A*, thyroglobulin; *B*, aldolase; *C*, bovine serum albumin; *D*, ovalbumin; *E*, chymotrypsinogen. Elution of free radioactivity is not shown on the graph. B. The chromatography was performed at room temperature and the elution buffer was Mg^{2+}-free. The figure shows the elution profiles of bound and free radioactivity contained in a control sample (□), and in EGS-treated samples (●) and an EGS-treated sample pretreated with EDTA (▲). In the latter and in the control samples, note that no bound radioactivity was detectable.

before treatment with EGS (Fig. 6A). As shown in Fig. 6B, the labeling of the two species was specific since it was abolished when an excess of unlabeled LVP was present at the stage of membrane incubation with [^{3}H]LVP. When electrophoresis was performed in the absence of DTT (Fig. 7), the ~50- and ~30-kDa labeled components were still detectable. In this case, a significant labeling was found associated to higher molecular weight compounds. As shown in Fig. 6A, the amount of radioactivity associated with the 50-kDa and 30-kDa subunits were similar under reduced or unreduced conditions. Therefore, it is unlikely that the higher molecular weight labeled species observed under unreduced conditions represent a homo or heterodimeric disulfide-linked subunit composition of the receptor. They probably result from [^{3}H]LVP unspecifically trapped with uncompletely undenaturated aggregated proteins.

Discussion

The results reported in this paper constitute part of an attempt to design a method allowing the physichochemical characterization and purification of vasopressin receptors. The main purpose of the present work was to define optimal conditions for the solubilization of the vasopressin-receptor complex in a stable form, the partial purification of this complex, and the formation of a covalent but easily cleavable linkage between the vasopressin molecule and its receptor.

The pig kidney cell line which was used does represent a convenient starting material for the physicochemical characterization and purification of adenylate cyclase-coupled vasopressin receptors. Indeed, these cells can be produced in large quantities. The maximal binding capacity of membranes purified from these cells by Percoll density gradient (3.4 pmol/mg of protein) is similar to the highest value reported by Crause and Fahrenholz (26) and Fahrenholz *et al.* (27) for purified bovine kidney membranes.

Previous studies have shown that vasopressin receptor solubilization by non-ionic detergents was accompanied by a marked reduction or a complete loss of the ability of the receptor to further bind vasopressin (21, 28). They also indicated that solubilization of preformed vasopressin-receptor complexes usually resulted in a partial dissociation of these complexes. Recently, it has been reported that V_1 vasopressin

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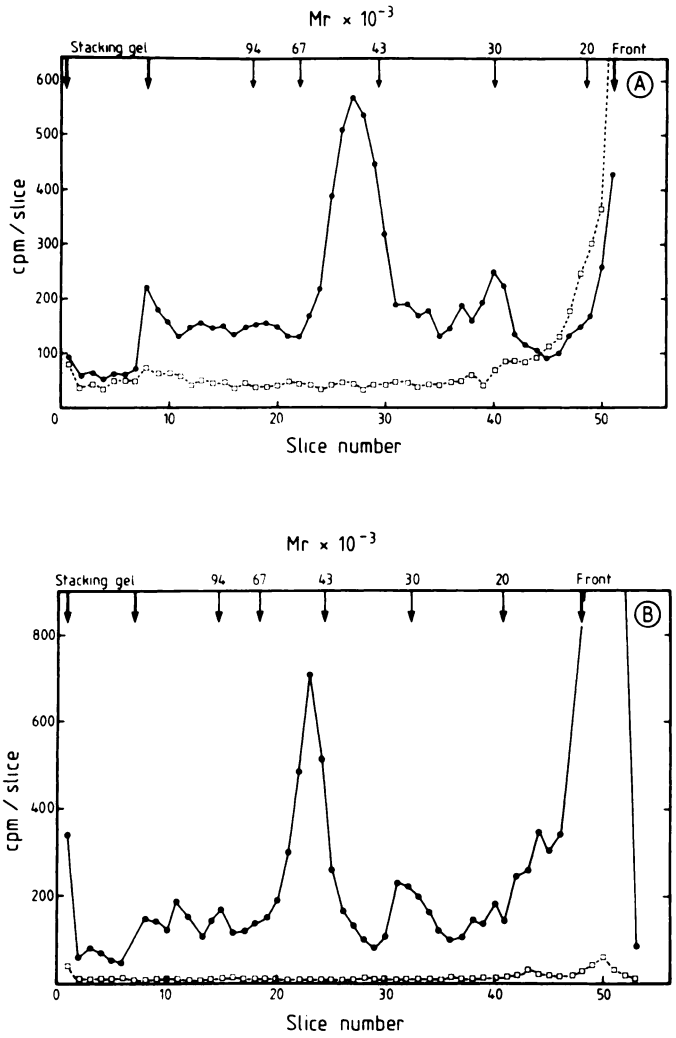

Fig. 6. SDS-polyacrylamide slab gel electrophoresis of partially purified [^{3}H]LVP-labeled vasopressin receptors treated with EGS. A. Dye-free prepurified [^{3}H]LVP-labeled vasopressin receptors were prepared, treated with EGS, and lyophilized as indicated under Materials and Methods. A control sample was treated with EDTA before cross-linking with EGS. The samples of lyophilized material corresponding to 150 μg of protein (24,000 cpm) were incubated in the presence of SDS (2%) and DTT (100 mM) for 1 hr at 30° and submitted to 12.5% polyacrylamide slab gel electrophoresis. The gel was sliced and the radioactivity of each slice (0.2 cm) was counted. ●, EGS-treated sample; □, control sample. ↓, the position on the gel of the molecular weight standard proteins. B. Plasma membranes were incubated with 10 nM [^{3}H]LVP in the absence or presence of 2 μM unlabeled vasopressin, solubilized, cross-linked, and lyophilized. The sample corresponding to 750 μg of protein (20,000 cpm) was treated and analyzed on SDS-PAGE as described for A. ●, total labeling; □, nonspecific labeling (presence of unlabeled vasopressin).

receptor was completely solubilized with 3% CHAPS and that their reconstitution into soybean phospholipid vesicules restored the binding capacity of the solubilized receptors (29). Thus, these results offer an alternative way of detecting unlabeled receptors during the steps of a purification procedure. We show here that detergents of the polyethoxy type allow, in the presence of glycerol, solubilization of vasopressin-receptor complexes with a high yield (83%) and minor dissociation (9%). Furthermore, the vasopressin-labeled solubilized material exhibited a surprisingly high stability upon storage at 4° (less than 6% dissociation within 24 hr). It behaved, upon steric exclusion chromatography, as an apparently homogeneous mo-

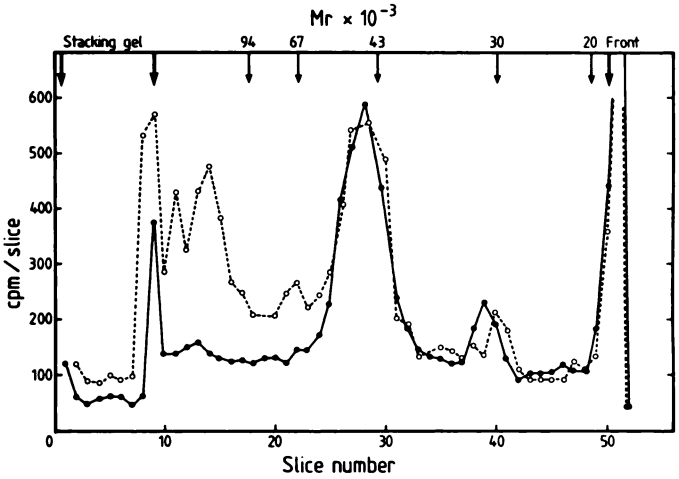

Fig. 7. SDS-polyacrylamide slab gel electrophoresis of partially purified [^{3}H]LVP-labeled vasopressin receptors treated with EGS: effect of DTT. The experiment was carried out as indicated in the legend to Fig. 6. Electrophoresis was performed in the presence (●) or absence (○) of 100 mM DTT.

lecular species. It would thus appear that solubilization of vasopressin receptors with $C_{12}E_8$ might be well suited for an attempt to partially purify the reversible complex formed between vasopressin and its receptor in LLC-PK$_1$ cell membranes. It is noticeable that the bulk of membrane protein is only partially solubilized, which leads to an enrichment of $C_{12}E_8$-soluble fractions in vasopressin-receptor complexes (6.4 pmol/mg of protein as compared to 3.1 pmol/mg of protein in purified membranes).

Among several methods which have been tested, chromatography on Cibacron-bleu-Sepharose columns appeared to be one of the most convenient methods to ensure partial purification of the reversible vasopressin-receptor complex with a high yield (84%) and minimal dissociation. The free dye was the only agent which eluted the [^{3}H]LVP-labeled vasopressin receptors from the Cibacron-blue-Sepharose column. We have no explanation for the observed high affinity of vasopressin receptors for Cibacron-blue F3GA. In any case, the procedure used for membrane preparation, solubilization, and partial purification ensured an overall 10-fold enrichment in vasopressin receptors as compared to the 10,000 × g pellet of the starting cell homogenate. This step was chosen to test the possibility of covalently linking vasopressin to its receptor by the use of a bifunctional cross-linking reagent.

We show that treatment with EGS of the [^{3}H]LVP-labeled vasopressin receptors in their membrane environment or in a solubilized and partially purified form markedly affected their stability in the presence of EDTA. Although EDTA rapidly and almost completely reversed vasopressin binding to untreated receptor, about 30% of the specifically bound vasopressin was insensitive to EDTA after EGS treatment. That EGS treatment also prevented the dissociation of bound [^{3}H]LVP in SDS-PAGE experiments clearly indicates that EGS had induced covalent cross-linking between the hormone and macromolecular membrane components. Taken together, these results suggest that the determination of EDTA-resistant vasopressin binding after EGS treatment does represent a convenient, reliable way of estimating the efficiency of cross-linking reaction.

The experiments with intact membranes suggest that most

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of the cross-linked vasopressin molecules were cross-linked to vasopressin receptors or molecules closely associated with these receptors. Indeed, EDTA-resistant vasopressin binding varied as a linear function of fractional receptor occupancy before EGS treatment. For a given fractional occupancy, it varied as a linear function of the maximal binding capacity of the membranes. In all circumstances, nonspecific cross-linking was negligible. EGS treatment of solubilized and partially purified vasopressin-receptor complexes did not affect its elution profile on steric exclusion HPLC. This observation suggests that there was no important cross-linking between the [^{3}H]LVP-labeled material and other components. This was not the case when cross-linking was performed on intact membranes.

SDS-PAGE of the[^{3}H]LVP-labeled and cross-linked material revealed two main components. The major one had a molecular weight of ≃50,000 and the minor one, a molecular weight of ≃30,000. The labeling of these two peaks was completely inhibited by LVP, suggesting that the labeling was specific. Using photoactivable vasopressin analogues, Fahrenholz *et al.* (6) detected a 30-kDa molecular species as the major component of V_2 vasopressin receptors from rat and bovine kidney membranes. It is tempting to consider that these 30-kDa components detected by photoaffinity labeling and cross-linking with EGS are homologous. It is worth noting that the photoaffinity labeling method also revealed a minor 58-kDa component (6). Whether this component is homologous to the major 50-kDa component detected by the cross-linking method remains an open question. The difference in molecular weight between these two components could be due to species differences in the origin of the receptor studied [a pig kidney cell line in the case of the present study and rat or bovine kidney in the study from Fahrenholz *et al.* (6)]. We cannot exclude that the ~50-kDa component revealed after EGS treatment of the LLC-PK$_1$ cell vasopressin receptor might reflect cross-linking of a receptor subunit with another molecule associated with the receptor. Furthermore, we cannot exclude that proteolysis could be responsible for the generation of two labeled peaks. Nevertheless, it seems unlikely that proteolysis occurred during the steps of prepurification because the ratio of the two peaks (50 kDa/30 kDa) was the same for the crude and prepurified cross-linked material. The preferential labeling of a 30-kDa component with a photoactivable vasopressin analogue (6) and that of a 50-kDa component with a cross-linking reagent should result from variations of the labeling yield with the nature of the subunit and the method of covalent labeling. In any case, the size of the vasopressin receptor subunit(s) appear(s) lower than the apparent size of the receptor determined by target size analysis (30) (108,000 and 95,000 for the rat and bovine kidney receptors, respectively) or hydrodynamic methods [80,000 in the case of the rat kidney receptor (31)]. The present data and those published by Fahrenholz *et al.* (6) may favor the hypothesis that V_2 vasopressin receptors have a polymeric structure.

To summarize we showed that the bifunctional reagent EGS allows covalent labeling of the vasopressin receptor from LLC-PK$_1$ cells with minimal nonspecific labeling and about a 10 times higher yield than achieved with photoactivable vasopressin analogues. This method might be helpful for further studies on vasopressin receptors.

Acknowledgments

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References

1. Jard, S. Vasopressin isoreceptors in mammals: relation to cyclic AMP-dependent and cyclic AMP-independent transduction mechanisms. *Curr. Top. Membr. Transp.* **18**:255–285 (1983).
2. Kirk, C. J., J. A. Creba, P. I. Hawkins, and R. H. Michell. Is vasopressin-stimulated inositol lipid breakdown intrinsic to the mechanism of Ca^{2+}-mobilization at V1 vasopressin receptors? *Prog. Brain Res.* **60**:405–411 (1983).
3. Jard, S., C. Roy, T. Barth, R. Rajerison, and J. Bockaert. Antidiuretic hormone-sensitive kidney adenylate cyclase. *Adv. Cyclic Nucleotide Res.* **5**:31–52 (1975).
4. Guillon, G., M. N. Balestre, B. Mouillac, G. Devilliers. Activation of membrane phospholipase C by vasopressin. A requirement for guanyl nucleotides. *FEBS Lett.* **196**:155–159 (1986).
5. Uhing, R. J., V. Prpic, H. Jiang, and J. H. Exton. Hormone-stimulated polyphosphoinositide breakdown in rat liver plasma membranes. Role of guanine nucleotides and calcium. *J. Biol. Chem.* **261**:2140–2146 (1986).
6. Fahrenholz, F., R. Boer, P. Crause, and M. V. Toth. Photoaffinity labelling of the renal V2 vasopressin receptor. Identification and enrichment of a vasopressin-binding subunit. *Eur. J. Biochem.* **152**:589–595 (1985).
7. Boer, R., and F. Fahrenholz. Photoaffinity labelling of the V1 vasopressin receptor in plasma membranes from rat liver. *J. Biol. Chem.* **260**:15051–15054 (1985).
8. Fahrenholz, F., E. Kojro, M. Muller, R. Boer, R. Lohr, and Z. Grzonka. Iodinated photoreactive vasopressin antagonists: labeling of hepatic vasopressin receptor subunits. *Eur. J. Biochem.* **161**:321–328 (1986).
9. Rogers, T. B. High affinity angiotensin II receptors in myocardial sarcolemmal membranes. Characterization of receptors and covalent linkage of ^{125}I-angiotensin II to a membrane component of 116,000 daltons. *J. Biol. Chem.* **259**:8106–8114 (1984).
10. Katoh, M., J. Djiane, and P. A. Kelly. Prolactin-binding components in rabbit mammary gland: Characterization by partial purification and affinity labeling. *Endocrinology* **116**:2612–2620 (1985).
11. Branca, A. A., P. M. Sluss, R. A. Smith, and L. E. Reichert. The subunit structure of the follitropin receptor: ehcmical cross-linking of the solubilized follitropin receptor complex. *J. Biol. Chem.* **260**:9988–9993 (1985).
12. Wood, C. L., and M. S. Dorisio. Covalent cross-linking of vasoactive intestinal polypeptide to its receptors on intact human by myoblasts. *J. Biol. Chem.* **260**:1243–1247 (1985).
13. Roy, C., and D. A. Ausiello. Characterization of (8-lysine) vasopressin binding sites on a pig kidney cell line (LLC-PK$_1$). Evidence for hormone-induced receptor transition. *J. Biol. Chem.* **256**:3415–3422 (1981).
14. Ji, T. H. The application of chemical cross-linking for studies on cell membranes and the identification of surface receptors. *Biochim. Biophys. Acta* **559**:39–69 (1979).
15. Abdella, P. M., P. K. Smith, and G. P. Royer. A new cleavable reagent for cross-linking and reversible immobilization of proteins. *Biochem. Biophys. Res. Commun.* **87**:734–742 (1979).
16. Pradelles, P., J. L. Morgat, P. Fromageot, M. Camier, D. Camier, D. Bonne, P. Cohen, J. Bockaert, and S. Jard. Tritium labelling of (8-lysine)-vasopressin and its purification by affinity chromatography on Sepharose-bound neurophysin. *FEBS Lett.* **26**:189–192 (1972).
17. Camier, M., D. Alazard, P. Cohen, P. Pradelles, J. L. Morgat, and P. Fromageot. Hormonal interactions at the molecular level: a study of oxytocin and vasopressin binding to bovine neurophysin. *Eur. J. Biochem.* **32**:207–214 (1973).
18. Atkinson, T., P. M. Hammond, R. D. Hartwell, P. Hughes, M. D. Scawen, R. F. Sherwood, D. A. P. Small, C. J. Bruton, M. J. Harvey, and C. R. Lowe. Triazine-dye affinity chromatography. *Biochem. Soc. Trans.* **9**:290–293 (1981).
19. Roy, C., A. Preston, and J. S. Handler. Insulin and serum increase the number of receptors for vasopressin in a kidney-derived line of cells grown in a defined medium. *Proc. Natl. Acad. Sci. USA* **77**:5979–5983 (1980).
20. Roy, C. Regulation of hormonal responsiveness in LLC-PK$_1$L cells grown in defined medium. *Am. J. Physiol.* **248**(Cell Physiol. **17**): C425–C435 (1985).
21. Roy, C., R. Rajerison, J. Bockaert, and S. Jard. Solubilization of the 8-lysine vasopressin receptor and adenylate cyclase from pig kidney plasma membranes. *J. Biol. Chem.* **250**:7885–7893 (1975).
22. Laemmli, U. K. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227**:680–685 (1970).
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
24. Peterson, G. L. A simplification of the protein assay. Method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**:346–356 (1977).
25. Lowe, C. R., and C. R. Pearson. Affinity chromatography on immobilized dyes. *Methods Enzymol.* **104**: 97–112 (1984).
26. Crause, P., and F. Fahrenholz. Affinities of reactive vasopressin analogues for bovine antidiuretic receptor. *Mol. Cell. Endocrinol.* **28**:529–541 (1982).
27. Fahrenholz, F., R. Boer, P. Crause, G. Fritzsch, and Z. Grzonka. Interactions

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of vasopressin agonists and antagonists with membrane receptors. *Eur. J. Pharmacol.* **100**:47–58 (1984).
28. Boer, R., P. Crause, and F. Fahrenholz. Solubilization of ligand-stabilized vasopressin receptors from plasma membranes of bovine kidney and rat liver. *Biochem. Biophys. Res. Commun.* **116**:91–98 (1983).
29. Dickey, B. F., J. B. Fishman, R. E. Fine, and J. Navaro. Reconstitution of rat liver vasopressin receptor coupled to guanine nucleotide-binding proteins. *J. Biol. Chem.* **262**:8738–8742 (1987).
30. Crause, P., R. Boer, and P. Fahrenholz. Determination of the functional molecular size of vasopressin isoreceptors. *FEBS Lett.* **175**:383–386 (1984).
31. Guillon, G., P. O. Couraud, D. Butlen, B. Cantau, and S. Jard. Size of vasopressin receptors from rat liver and kidney. *Eur. J. Biochem.* **111**:287–294 (1980).

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