# Direct Identification of an Extracellular Agonist Binding Site in the Renal $V_2$ Vasopressin Receptor†

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ABSTRACT: To purify the renal $V_2$ receptor and identify domains involved in hormone binding, photoaffinity labeling of the membrane-bound bovine $V_2$ receptor with a tritium-labeled photoreactive vasopressin agonist was performed. The labeled 30 000 $M_r$ protein was purified to homogeneity by anion-exchange chromatography, isoelectric focusing, gel filtration, gel electrophoresis, and reversed-phase HPLC. N-terminal sequencing showed that the isolated protein which contains the covalently bound hormonal ligand, represents an N-terminal truncated bovine $V_2$ receptor. The purified labeled $V_2$ vasopressin receptor protein was digested with $V_8$ protease, and peptide fragments were isolated. Protein microsequencing and comparison with the cDNA sequence of a cloned PCR product identified two extra- and two intracellular peptides of the bovine $V_2$ receptor. Radioactivity was incorporated into two amino acid residues localized in the second extracellular domain. Our results indicate that this extracellular domain is involved in peptide agonist binding of the $V_2$ receptor.

The biological actions of the neurohypophyseal nonapeptide vasopressin are mediated by at least two subtypes of vasopressin receptors with different ligand specificities and different second messenger systems (Michell et al., 1979; Jard, 1983). Molecular cloning of the rat $V_1$ receptor (Morel et al., 1992) and $V_2$ receptors from three mammalian species (Birnbaumer et al., 1992; Lolait et al., 1992; Gorbulev et al., 1993) has shown that these receptors are members of the G-protein-coupled receptor family that is characterized by seven putative transmembrane helices. At present, functional domains and posttranslational modifications of vasopressin receptor proteins have not been characterized. Knowledge of the hormone-binding site of the $V_2$ receptor would be of particular interest. This receptor subtype is located mainly in the distal collecting ducts, is coupled to the activation of the adenylate cyclase system (Chase & Aurbach, 1968), and mediates the antidiuretic action of vasopressin (Orloff & Handler, 1967). Mutations of the $V_2$ receptor were detected by several authors in patients with nephrogenic diabetes insipidus (Davies, 1993), an X-linked inherited disease which is characterized by unresponsiveness of the $V_2$ receptor system for the antidiuretic hormone. These mutations could impair either hormone binding (Luzius et al., 1992) or hormone-induced signal transfer (Rosenthal et al., 1993). Furthermore, elucidation of the hormone-binding site could facilitate the design of specific $V_2$ agonists and antagonists (Manning & Sayer, 1991) for the treatment of disturbances in the vasopressin-regulated salt and water balance (Schrier, 1990).

The localization of the ligand-binding domain in the seven-transmembrane hormone receptor family was studied by site-directed mutagenesis, deletion analysis, and construction of chimeric receptors (for a review see Ostrowski et al. (1992)). As these mutations might affect hormone binding indirectly by changing the conformation of a receptor or its expression in the plasma membrane, we decided to use a more direct protein chemical approach: the covalent attachment of a radiolabeled hormonal ligand to the hormone receptor and the identification of labeled amino acids. Purification of the renal $V_2$ vasopressin receptor protein has been prevented until now by the detergent sensitivity of this receptor, resulting in either a significant reduction or a total loss of hormone-binding activity (Boer et al., 1983; Aiyar et al., 1989). For this reason, the strategy which we developed required covalent labeling of the $V_2$ receptor in the plasma membrane with a tritium-labeled photoactivatable ligand followed by solubilization and purification of the labeled receptor by conventional methods. As the covalent reaction between a photoreactive hormonal ligand and the receptor often proceeds with a low yield, it is important to note that several affinity-labeled hormone receptors exhibit the same chromatographic behavior as the corresponding unlabeled receptors (Duong et al., 1989; Usui et al., 1990).

As starting material for the purification of the renal $V_2$ receptor, membranes derived from bovine kidney medulla were chosen. These membranes contain a $V_2$ receptor density which is severalfold higher than that described for membranes derived from other mammalian species (Crause & Fahrenholz, 1982). In this report, we describe the purification of the bovine $V_2$ receptor after labeling with a photoreactive vasopressin agonist and the isolation of $V_2$ receptor peptide fragments. Furthermore, an extracellular domain of the renal $V_2$ receptor which is involved in hormone agonist binding is identified by protein microsequencing.

## MATERIALS AND METHODS

*Materials.* The radioactive photoreactive vasopressin agonist [$^3$H][Mpa$^1$,Lys(Apa)$^8$]VP (specific radioactivity 52.7 Ci/mmol) was prepared by reaction of $^3$H-labeled 1-deamino-[8-lysine]vasopressin with methyl 4-azidobenzimidate hydrochloride and purified by HPLC as previously described (Fahrenholz et al., 1988).[1] All other chemicals were from commerical sources.

*Membrane Preparation.* Plasma membranes from bovine kidney medulla were prepared by differential centrifugation followed by Percoll density gradient centrifugation as described previously (Crause & Fahrenholz, 1982). Typically for a

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membrane preparation, the papillas from 10 kidneys were isolated and 110 mg of plasma membranes was obtained. Membrane preparations obtained by this procedure had a specific binding capacity of 2–6 pmol of [$^3$H]AVP/mg of protein. The preparative purification of the affinity-labeled $V_2$ receptor was performed with 3.33 g of membranes isolated from 300 bovine kidneys; membranes were stored at −70 °C.

*Preparative Photoaffinity Labeling of Membranes.* Frozen plasma membranes in 0.25 M sucrose buffer were thawed and centrifuged at 50000*g* for 20 min at 4 °C. Membranes (about 3.33 g) containing 16 nmol of receptor were resuspended in 1600 mL of binding buffer (50 mM Hepps, pH 8.4, 5 mM $MgCl_2$) to obtain a $V_2$ receptor concentration of $1 \times 10^{-8}$ M and were incubated with $5 \times 10^{-8}$ M tritium-labeled photoreactive ligand for 30 min at 30 °C. The suspension was cooled in an ice bath for 15 min; then membranes were separated from unbound ligand by centrifugation at 50000*g* for 30 min at 4 °C. After resuspension of the membrane pellet in 1600 mL of ice-cold binding buffer containing 5 mM *p*-aminophenylalanine as a scavenger, aliquots of the mixture were exposed in a quartz tube (8-mL volume) to three 1-ms flashes produced in an apparatus for high energy ultraviolet irradiation (Frimmer & Ziegler, 1986). After irradiation, plasma membranes were collected by centrifugation at 4 °C for 30 min at 50000*g*. The pelleted membranes were resuspended in water to a protein concentration of 10 mg/mL and used immediately for solubilization.

*Membrane Solubilization.* After photoaffinity labeling, membranes (about 3.33 g) were solubilized in 665.5 mL (5 mg of protein/mL) of 50 mM Hepps buffer, pH 8.3, containing 1% digitonin, 0.1% sodium cholate, 5 mM $MgCl_2$, 100 mM NaCl, 5 mM EDTA, 10 μg/mL trypsin inhibitor, and 5 μg/mL each of antipain, leupeptin and pepstatin. The suspension was gently shaken in a water bath for 30 min at 30 °C and then centrifuged at 50000*g* for 1 h at 4 °C. The resulting supernatants were combined and used immediately for further purification.

*Purification of $V_2$ Vasopressin Receptor from Bovine Kidney.* The column chromatographic steps were performed with a FPLC system (Pharmacia) at 4 °C; gel filtration in SDS buffer was carried out at room temperature. Preparation of digitonin (Sigma) for all buffer systems was as described (Cubero & Malbon, 1984). Briefly: 5% digitonin solution in water was heated at 95-100 °C for 30 min, and after standing for 48 h at 25 °C, the solution was filtered through a 0.22-μm filter (Millipore).

*Sepharose Q Column Chromatography.* The column was equilibrated with buffer A containing 10 mM Bis-Tris, pH 7.0, 20 mM NaCl, and 0.2% digitonin. The detergent extract of bovine kidney membranes was diluted with 10 mM Bis-Tris buffer, pH 7.0, to a volume of 3327 mL and applied on the Sepharose Q column (16 × 100 mm, Pharmacia) in three aliquots. Proteins were eluted with a gradient of buffer A containing 1 M NaCl. The column eluate was fractionated (5-mL fractions), and 10-μL aliquots were taken for scintillation counting. The radioactive fractions 24–34 were collected.

To concentrate the probe, pooled fractions from three runs were collected, diluted 1:10 with 10 mM Bis-Tris, pH 7.0, and 0.2% digitonin, applied again on the Sepharose Q column, and eluted as described above.

*Liquid-Phase Isoelectric Focusing (Rotofor).* The fractions (45 mL) pooled after elution of the second Sepharose Q column were diluted to a final volume of 200 mL containing 1.5% digitonin, 2% ampholytes, pH range 3–10 (Bio-Rad), 1 mM DTT, 50 mM NaCl, 2.5 mM Bis-Tris, pH 7.0, and 20% glycerol. This solution was loaded in 50-mL aliquots into the Rotofor cell (Bio-Rad) for four consecutive runs. Focusing was carried out at 12-W constant power for 4–4.5 h at 4 °C. The initial conditions were 250 V and 50 mA. (At equilibrium, the values were 850 V and 13 mA). Electrolytes in the anode and cathode chambers were 0.1 M $H_3PO_4$ and 0.1 M NaOH, respectively. Twenty fractions were collected, and their protein concentrations, pH values, and radioactivities were measured. Protein fractions 8–10 containing radioactivity and having pH values between 5.5 and 6.8 were pooled from four runs and refractionated. To 25 mL of the sample solution were add 5 mL of glycerol and 20 mL of 1.5% digitonin to a final volume of 50 mL. Refractionation was done without additional ampholytes. Focusing was carried out for 1 h and 40 min under the same conditions as described above. (At equilibrium, the values were 1350 V and 8 mA.) Radioactive protein fractions 5–11 with pH values between 5.8 and 6.5 were pooled and applied on gel filtration column.

*Superdex 200 Column Chromatography.* The fractions (15 mL) pooled after isoelectric focusing were applied to a Superdex 200 gel filtration column (2.0 × 60 cm) equilibrated and eluted with column buffer containing 10 mM Bis-Tris, pH 7.0, 100 mM NaCl, and 2% SDS at room temperature. The column eluate was fractionated (10-mL fractions), and 50-μL aliquots were taken for scintillation counting. Protein fractions 14–16 containing radioactivity were pooled and stored at −20 °C for further purification.

*High-Performance Electrophoresis Chromatography (HPEC).* For each HPEC run, protein (500 μg) from 2 mL of protein solution obtained after chromatography on Superdex-200 was precipitated with chloroform/methanol (Wesch & Flügge, 1984). The protein pellet was incubated for 10 min with 10 μL of 10% SDS; then 140 μL of sample buffer (15 mM tris-phosphate, pH 7.5, 2% SDS, 0.4% β-mercaptoethanol, 30% glycerol) was added, and the sample was incubated for 1 h at room temperature. After centrifugation for 10 min at 10000*g*, 150 μL of the supernatant was applied on a calibrated acrylamide tube gel in the HPEC system.

Protein separation was performed with a tris-phosphate–SDS buffer system at pH 7.5 according to the protocol of Applied Biosystems; 3% stacking and 6% separation polyacrylamide tube (3.5 × 100 mm) gels were used. The gels were prerun for 60 min with a constant current of 0.5 mA and then for 240 min with a current gradient from 1.5 to 2.5 mA. Calibrating runs with standard proteins and separating runs were performed for 600 min under the following conditions: 60 min with a constant current of 0.5 mA, then 440 min with a current gradient from 1.5 to 2.5 mA, finally 100 min with a constant current of 2.5 mA. All electrophoresis runs were performed at 8 °C. Proteins were eluted from the gel at a flow rate of 50 μL/5 min and detected at 220 nm. Protein fractions were collected at 4 °C: 50-μL fractions were collected, 1-μL aliquots of each fraction were analyzed for protein composition (SDS–PAGE, mini-gels, silver staining), and 10-μL aliquots were taken for scintillation counting.

*Reversed-Phase HPLC.* The pooled radioactive fractions after HPEC (1.5 mL) were applied in 500-μL aliquots to a $C_4$ reversed-phase column (250 × 4.6 mm, 5 μm, Vydac). HPLC was performed with the buffer system 0.1% TFA in

[1] Abbreviations: [$^3$H][Mpa$^1$,Lys(Apa)$^8$]VP, [1-(3-mercaptopropionic acid), 2-(3,5-ditritiotyrosine),8-($N^6$-((4-azidophenyl)amidino)lysine)]-vasopressin; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPEC, high-performance electrophoresis chromatography; TFA, trifluoracetic acid.

water (buffer A) and 0.1% TFA in 2-propanol/acetonitrile (2:1) (buffer B) using a linear gradient of buffer B from 0 to 100% over 60 min. A flow rate of 1.5 mL/min was maintained. The elution of protein was monitored by UV absorbance at 220 nm. Fractions of 1.5 mL were collected, and 150-$\mu$L samples of each fraction were taken for scintillation counting.

*Digestion of Labeled $V_2$ Vasopressin Receptor with Endoproteinase Glu-C.* A portion of the HPLC eluate with about 70 pmol of purified protein was concentrated in an Eppendorf tube by evaporation in a Speed Vac centrifuge (Savant) to a volume of 50 $\mu$L; then 5 $\mu$L of 10% octyl glucoside and 500 $\mu$L of water were added. Evaporation with 500 $\mu$L of water was repeated three times to completely remove TFA. Finally, the sample was dried and resuspended in 50 $\mu$L of digestion buffer containing 25 mM ammonium carbonate, pH 7.8, 1% octyl glucoside, and 0.5 $\mu$g of endoproteinase Glu-C (Boehringer, Mannheim, Germany). The digestion was performed at room temperature overnight at a protease to protein ratio of about 1:5.

*Reversed-Phase HPLC Separation of Receptor Fragments.* The enzyme-treated sample was dried by Speed Vac evaporation, resuspended in 50 $\mu$L of 0.1% TFA, and loaded on a $C_4$ reversed-phase microbore HPLC column (Vydac, 100 × 2.1 mm) equilibrated with 0.1% TFA in water (buffer A). Peptides were eluted over 100 min with the following gradient of 0.085% TFA/acetonitrile (buffer B) at 150 $\mu$L/min flow: 5 min, 0% B; 60 min, 35% B; 90 min, 80% B; 100 min, 100% B. The elution profile was monitored by absorbance at 214 nm. The relevant peak fractions were stored at 4 °C and prepared for amino acid sequencing.

*N-Terminal Sequence Analysis.* Pure protein and peptide fractions obtained after HPLC were concentrated in a Speed-Vac centrifuge (Savant) nearly to dryness, solubilized in 30 $\mu$L of 100% TFA, and applied on a glass filter disk derivatized with Bio Brene Plus. Sequencing was carried out on a pulsed-liquid sequencer (475, Applied Biosystems) which was equipped with an on-line analyzer for phenylthiohydantoin (PTH) derivatized amino acids. Forty percent of the PTH-amino acid which remained in the fraction collector was used for radioactivity counting.

*Determination of Receptor and Protein Content and SDS–PAGE.* The recovery of $V_2$ vasopressin receptor protein with $M_r$ = 30 000 was determined after each purification step by scintillation counting of fractions containing protein-bound [$^3$H][Mpa$^1$,Lys(Apa)$^8$]VP and by tube SDS–PAGE (11% acrylamide gel). After electrophoresis, gels were sliced for liquid scintillation counting and the amount of radioactivity in the $M_r$ 30 000 protein band was specified. Protein concentration was determined by the method of Bradford (Bradford, 1976) and fluorescamin (van Frank, 1975) with bovine serum albumin as standard. The protein content of the final receptor preparation was estimated both by N-terminal sequencing and by SDS–PAGE (11%) followed by silver staining (Wray et al., 1981). Gel electrophoresis was performed according to Laemmli (1970) with either 11% tube or slab gels.

*Receptor Binding Assays.* Plasma membranes from bovine kidney containing 80–100 $\mu$g of protein were incubated with [$^3$H]vasopressin or with $^3$H-labeled peptides for 30 min at 30 °C. The binding assay and data analysis were performed as described previously (Fahrenholz et al., 1984).

*Polymerase Chain Reaction.* Two degenerate oligonucleotides corresponding to transmembrane domains (TM) 2 and 6 were designed on the basis of the cDNA sequences of the cloned vasopressin and oxytocin receptors as described (Gorbulev et al., 1993). The 5′-primer was 5′-TTCCA(A/G)GT(G/A)(C/T)TGCC(C/G)CAGCT-3′, and the 3′-primer was 5′-GAAGAA(G/A)GG(T/C)GCCCAGCA(C/A)A-3′. The mRNA was isolated from bovine kidney by the standard guanidinium thiocyanate method and oligo(dT)-cellulose chromatography. Oligo(dT)-primed cDNA was synthesized with superscript reverse transcriptase (Gibco, BRL) and used in PCR as a template under the following conditions: 30 cycles, 94 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min. The PCR product was gel-purified, cloned into pGEM-7Zf(+), and sequenced.

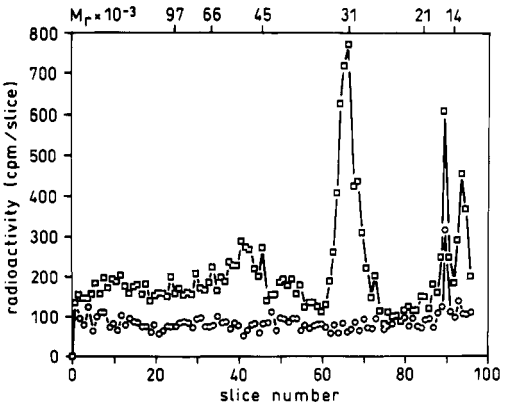


FIGURE 1: Photoaffinity labeling of bovine kidney membranes with [$^3$H][Mpa$^1$,Lys(Apa)$^8$]VP. Membrane protein (1 mg) with a receptor density of 3 pmol/mg of protein was incubated with 0.025 $\mu$M photoreactive ligand in the absence of AVP (□) or in the presence of 2.5 $\mu$M AVP (○). After irradiation, membrane proteins were subjected to electrophoresis on SDS–PAGE and gels were sliced for counting.

## RESULTS

*Photoaffinity Labeling.* Photoreactive analogues of 1-deamino[8-lysine]vasopressin containing a photoreactive aryl azido group at the side chain of Lys$^8$ retain affinity in the nanomolar range for the bovine $V_2$ receptor. After photoactivation of these tritium-labeled photoreactive analogues, a membrane protein from bovine kidney with an apparent molecular weight of $M_r$ 30 000 was preferentially and specifically labeled and a protein with an $M_r$ value of 58 000 was labeled with much less efficiency (Fahrenholz et al., 1985, 1988).

For the purification of the labeled bovine $V_2$ receptor protein with $M_r$ of 30 000, photoaffinity labeling on a preparative scale with the (azidophenyl)amidino (Apa) analogue of 1-deamino[8-lysine]vasopressin ([Mpa$^1$,Lys(Apa$^8$)]VP) was performed. This ligand retains the positive charge in position 8 and the highest binding affinity ($K_D$ = 1.8 nM) of the photoreactive arylazido vasopressin analogues. Unspecific labeling with this ligand is low (Figure 1), and the yield of covalent incorporation into the renal $V_2$ receptor protein with $M_r$ of 30 000 was estimated to be 3–5% of the receptor protein. After photoaffinity labeling of 3.33 g of plasma membranes isolated from 300 bovine kidneys, the labeled 30 000 $M_r$ receptor protein was used as a tracer for purification of the $V_2$ receptor.

*Solubilization.* The efficiency of solubilization of the photoaffinity-labeled vasopressin receptor was determined for various nonionic detergents: after chloroform/methanol precipitation of the detergent-solubilized photolabeled membranes, proteins were separated by SDS gel electrophoresis and the amount of $^3$H-labeled 30 000 $M_r$ protein was determined as described under Material and Methods. These values were compared with that obtained after SDS gel electrophoresis of photolabeled membranes. The following nonionic detergents efficiently solubilized the affinity-labeled vasopressin receptor: 2% Triton X-100 (35.5%); 1% dodecyl

Table I: Purification of Labeled $V_2$ Vasopressin Receptor Protein from Bovine Kidney Membranes

| step | protein (mg) | receptor (pmol) | specific radioactivity (cpm/μg) | purification (fold) | yield (%) |
|---|---|---|---|---|---|
| membrane | 3327 | 16052 | 2.5[a] | | 100 |
| extract | 1413 | 12842 | 5.3[a] | 2.12 | 80 |
| Sepharose Q | 166.8 | 7320 | 17.0[a] | 6.8 | 46 |
| Rotofor cell | 21.5 | 3660 | 71.4[b] | 28.6 | 23 |
| Superdex 200 | 8.0 | 1998 | 185.6[b] | 74.4 | 13 |
| HPEC | 1.1 | n.d. | n.d. | n.d. | n.d. |
| HPLC | ≈0.003 | ≈100[c] | n.d.[d] | ≈6000–10 000 | ≈0.6 |

[a] Calculated from the amount of radioactivity obtained after SDS-PAGE in 30 000 molecular weight range. [b] Calculated from the amount of radioactivity obtained in pooled fractions. [c] Determined from N-terminal amino acid sequence analysis and from SDS–PAGE. [d] Due to the instability of the hormone receptor complex during purification, the specific radioactivity was only roughly 10% of the theoretical calculated value (≈150 000 cpm for incorporation yield of 3%).

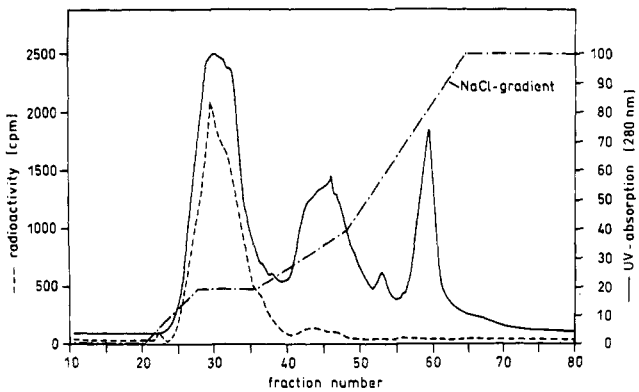


FIGURE 2: Ion-exchange chromatography of the photoaffinity-labeled $V_2$ receptor. The Sepharose Q column was equilibrated with column buffer at 4 °C. After binding of proteins, elution was performed with column buffer containing 1 M NaCl. The column eluate was fractionated (5-mL fractions), and 10-μL aliquots were taken for scintillation counting. Radioactive fractions were tested for $M_r$ = 30 000 protein by SDS gel electrophoresis as described under Material and Methods. The radioactive fractions 32–34 containing the labeled protein were collected.

β-D-maltoside (48%); 1% digitonin (63.3%). Solubilization was further increased in the presence of 100 mM NaCl. The $V_2$ vasopressin receptor was solubilized on a preparative scale in binding buffer containing 1% digitonin, 0.1% sodium cholate, 100 mM NaCl, and proteinase inhibitors, at a membrane protein concentration of 5 mg/mL. With these conditions, approximately 80% of the labeled $V_2$ receptor and 35–40% of the total membrane proteins were solubilized, yielding a 2-fold enrichment of the receptor protein (Table I).

*Purification.* The purification strategy and the results are summarized in Table I. The first two steps involved separation according to charge differences. Sepharose Q anion-exchange chromatography separated the photoaffinity-labeled protein from most of the free ligand. Figure 2 shows a typical elution profile obtained with a NaCl gradient. The affinity-labeled vasopressin receptor eluted at 200 mM NaCl concentration, whereas the free ligand was not adsorbed to the column. By two consecutive separations on Sepharose Q, the protein sample was concentrated from a volume of 3327 mL to 45 mL and the $V_2$ receptor protein was enriched 3.2-fold with 57% recovery. Liquid-phase preparative isoelectric focusing (in a Rotofor cell) was used for further enrichment of the $V_2$ vasopressin receptor protein. With this carrier-free method, the protein could be easily recovered with a good yield from the solution after focusing. Digitonin at a concentration of 1.5% in solution was necessary to keep the proteins in a soluble form. The labeled vasopressin receptor protein focused within

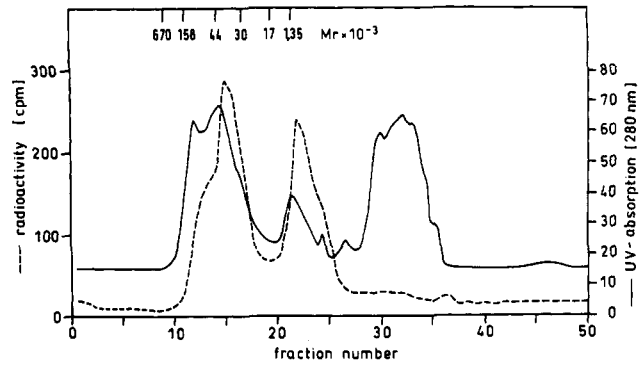


FIGURE 3: Gel filtration chromatography of the photoaffinity-labeled $V_2$ receptor. Radioactive fractions after preparative isoelectric focusing were applied on a Superdex 200 column and eluted with buffer containing 2% SDS at room temperature as described under Materials and Methods. The column eluate was fractionated (10-mL fractions), and 80-μL aliquots were taken for scintillation counting. Radioactive fractions were tested for $M_r$ = 30 000 protein by SDS gel electrophoresis as described under Materials and Methods. Radioactive fractions 14–16 containing the labeled protein were pooled.

a pH range of 5.8–6.5. Isoelectric focusing in the Rotofor cell substantially removed low molecular weight proteins and some dominant proteins with a molecular weight between 25 000 and 35 000, yielding a 4.2-fold purification of the labeled $V_2$ vasopressin receptor protein.

A strongly denaturating detergent was necessary to obtain protein resolution and an acceptable recovery on a molecular sieve (Superdex 200) column. During gel filtration in digitonin, formation of protein aggregates resulted in the elution of the receptor protein in the void volume of the column (data not shown). Therefore, in the gel filtration step, the detergent digitonin was replaced by SDS. Figure 3 shows the elution profile of the protein mixture from the Superdex 200 column. Two radioactive peaks were observed, one in a small molecular weight range representing free ligand and the second in a molecular weight range between 30 000 and 44 000 representing protein-bound ligand. The fractions from the second radioactive peak were pooled and used for further purification.

High-performance electrophoresis chromatography (HPEC), a tube SDS–PAGE system with continuous elution and on-line UV detection (Sheer et al., 1990), was an efficient method for semipreparative purification of the labeled $V_2$ vasopressin receptor. The conditions for HPEC purifications were optimized in analytical experiments either with digitonin-solubilized photolabeled plasma membranes or with the eluate from Sepharose Q columns. The enrichment obtained from several runs was between 20- and 23-fold, and the recovery of total membrane protein was between 70 and 75%. In semipreparative experiments, the $V_2$ receptor enriched fraction from the fourth purification step (gel filtration) was applied in aliquots of 500 μg of protein to the HPEC system. The typical protein elution profile and the distribution of radioactivity from a 6% polyacrylamide tube gel are shown in Figure 4A. The main radioactive peak corresponds by comparison with standard proteins to a molecular weight of about 30 000. The fractions from semipreparative SDS–PAGE were analyzed by analytical SDS–PAGE and silver staining (Figure 4B). Radioactive fractions containing 30 000 $M_r$ proteins (fractions 24–27) from 16 HPEC runs were combined. After semipreparative SDS–PAGE, only about 10% of the totally applied radioactivity was recovered in fractions containing proteins with $M_r$ of 30 000. The total recovery of radioactivity from all fractions after an HPEC run was between 80 and 90%. As protein aggregates with higher molecular weight

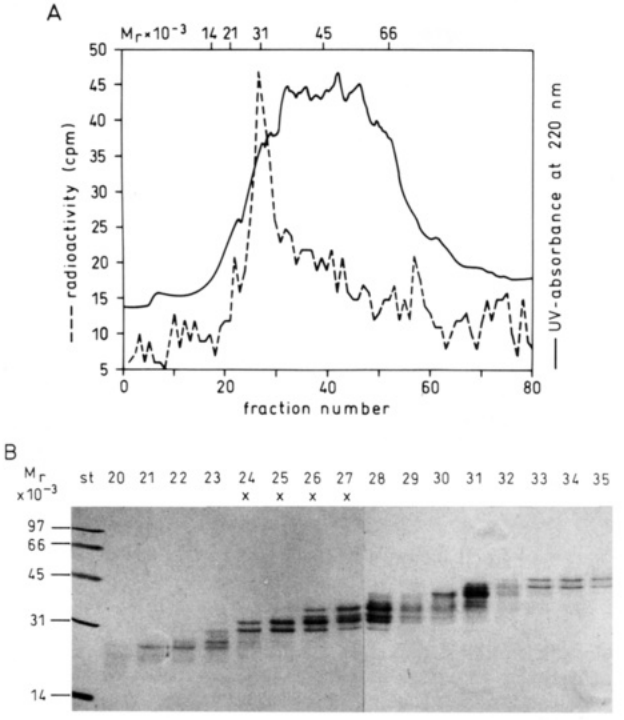


FIGURE 4: Elution profile, radioactivity distribution, and protein pattern after semipreparative SDS–PAGE (HPEC). (A) 500 μg of protein enriched by gel filtration was applied on the tube gel (3.5 × 100 mm). Protein eluate was monitored continuously by UV detection, and 10-μL aliquots of each fraction were taken for scintillation counting. Tubes were calibrated by standard proteins. (B) SDS–PAGE analysis of fractions 20–35 after an HPEC run. Fractions containing radioactivity are labeled by ×. Proteins were visualized by silver staining.

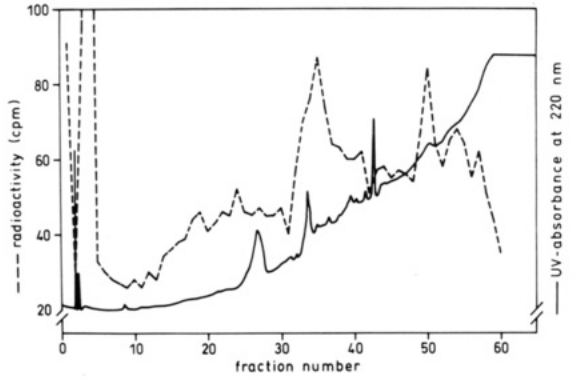


FIGURE 5: Reversed-phase HPLC. The $C_4$ column (Vydac 250 × 4.6 mm) was equilibrated with 0.1% TFA/$H_2O$ (solvent A). Solvent B was 0.1% TFA in 2-propanol/acetonitrile (2/1). Radioactive fractions from HPEC were applied and eluted with a linear gradient from 0 to 100% B, at a flow rate of 1.5 mL/min. Fractions of 1.5 mL were collected, and 150-μL aliquots were taken for scintillation counting.

were not found, this observation suggests a partial instability of the covalent complex between receptor and radioactive ligand during 10-h preparative gel electrophoresis.

Finally, the labeled vasopressin receptor protein was purified to homogeneity by HPLC on a reversed-phase $C_4$ column. The distribution of radioactivity and UV absorption after the HPLC run are shown in Figure 5. While the first radioactive peak corresponds to the free ligand, two other radioactive peaks represent covalent ligand–protein complexes. Analysis of the protein fractions containing radioactivity by SDS–PAGE showed that the main radioactive fraction contains a homogeneous protein with a molecular weight of ≈31 000 (Figure 6). The final amount of the purified $V_2$ receptor protein was estimated from SDS–PAGE and the N-terminal

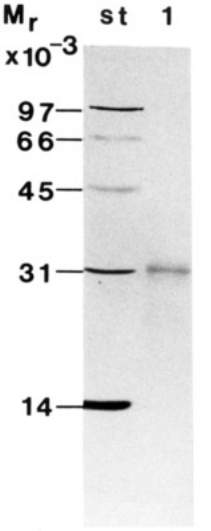


FIGURE 6: SDS–PAGE analysis of purified photoaffinity-labeled vasopressin receptor. 15-μL aliquots of the fraction with highest radioactivity (no. 35) from HPLC were subjected to 11% SDS–PAGE, and the gel was silver stained (st: standard proteins).

sequence analysis to be roughly 100 pmol. As summarized in Table I, the labeled $V_2$ vasopressin receptor was purified about 6000–10000-fold from bovine kidney membranes with a yield of ~0.6%. Purification and recovery factors of the intermediate purification steps are underestimated because free radioactive ligand was observed in each eluate.

*N-Terminal Sequence Analysis.* To identify the purified protein, N-terminal sequence analysis with 20–25 pmol of labeled protein was performed. The following amino acid sequence was obtained: -X-X-Pro-Gln-Leu-Ala-Trp-Asp. The identification of the first two amino acids was not possible because of contaminations in the first two cycles of Edman degradation. The next five amino acids are identical with a sequence found exclusively in the cloned human, rat, and pig $V_2$ receptor. This pentapeptide sequence is at the C-terminus of the nonapeptide Phe-Gln-Val-Leu-Pro-Gln-Leu-Ala-Trp, which is conserved in the three cloned $V_2$ receptors. It is located at the transition between the second transmembrane domain and the second extracellular domain (Figure 7B). The N-terminal amino acid of the labeled bovine $V_2$ receptor protein corresponds to $Val^{93}$ of the human homologue (Birnbaumer et al., 1992). The protein sequence information clearly shows that the purified photoaffinity-labeled protein represents a truncated bovine $V_2$ receptor which lacks part of its N-terminus. This result also demonstrates that the hormone-binding domain responsible for interaction with the photoreactive vasopressin analogue is localized in the N-terminal truncated $V_2$ vasopressin receptor with an apparent molecular weight of 30 000.

*Purification and Sequencing of $V_2$ Vasopressin Receptor Peptide Fragments after Endoproteinase Glu-C Digestion.* To further prove the identification of the purified protein and to localize the site of incorporation of the hormone analogue, peptide fragments of the labeled $V_2$ vasopressin receptor were generated by cleavage with endoproteinase Glu-C ($V_8$ protease from *Staphylococcus aureus*). $V_8$ protease cleaves in ammonium carbonate buffer at pH 7.8 peptide bonds at the carboxyl side of glutamic acid residues. This cleavage strategy was chosen to obtain a few large peptide fragments from the limited amount of receptor protein to facilitate the separation of fragments and their microsequence analysis. After proteolytic digestion of the photoaffinity-labeled $V_2$ receptor, the resulting peptide mixture was separated by microbore reversed-phase HPLC (Figure 7A). The fractions corresponding to

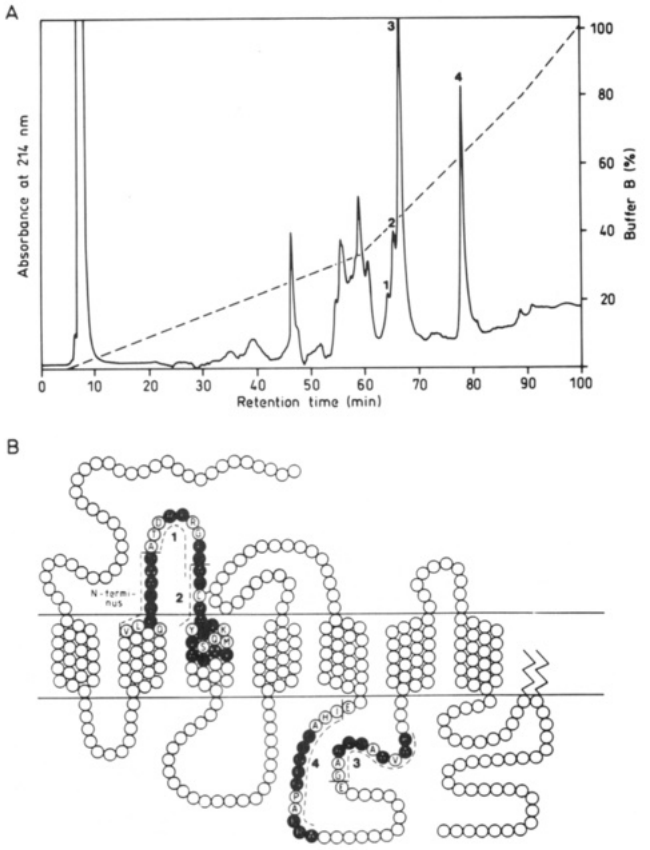


FIGURE 7: Partial protein sequences in the bovine $V_2$ receptor. (A) Separation of receptor fragments after endoproteinase Glu-C digestion by reversed-phase HPLC. Chromatographic conditions: $C_4$ column (Vydac 2.1 mm × 100 mm); solvent A 0.1% TFA/$H_2O$, solvent B 0.085% TFA/acetonitrile; flow rate 150 μL/min. (B) Localization of peptide fragments 1–4 and of the N-terminus of the truncated bovine $V_2$ receptor in a two-dimensional model of the $V_2$ receptor. Residues identified by protein sequencing are shown by white letters on a black background.

peaks of the UV absorption were collected, and the isolated peptides were used for amino acid sequence analysis.

The small amounts of purified fragment peptides prevented the unequivocal identification of all amino acid residues during protein sequence analysis. For better identification and characterization of the receptor fragments, the cDNA sequence between transmembrane regions 2 and 6 of the bovine $V_2$ vasopressin receptor was determined from a cloned PCR product. This product extends from residues 99 to 288 of the human $V_2$ receptor homologue; 89% of the amino acid residues were identical with those of the human $V_2$ receptor. Comparison of protein and cDNA sequences from the same species led to the identification of four fragments of the $V_2$ bovine vasopressin receptor obtained by endoproteinase Glu-C cleavage (Table II). The two peptides 3 and 4 represent receptor fragments obtained by cleavage after glutamic acid residues of the vasopressin receptor polypeptide. Both peptides are located in the third cytoplasmic domain of the $V_2$ vasopressin receptor (Figure 7B). Peptides 1 and 2 were generated by cleavage of Asp–Ala bonds. Cleavage at the carboxyl site of aspartic acid by $V_8$ protease in ammonium carbonate buffer has been observed when a small amino acid residue is adjacent to an aspartic acid in a polypeptide chain (Houmard & Drapeau, 1972). Both peptides 1 and 2 are located in the second extracellular domain of the $V_2$ vasopressin receptor (Figure 7B).

To identify those amino acid residues of the receptor protein, to which the hormonal ligand is covalently bound, 20 cycles of Edman degradation were performed for each peptide and

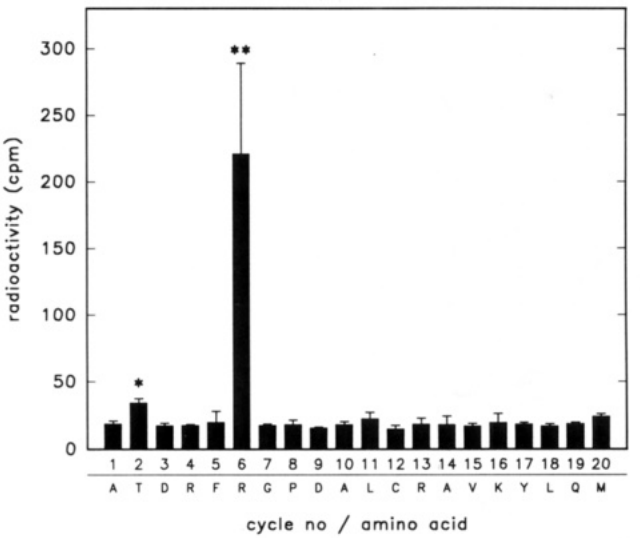


FIGURE 8: Radioactivity distribution after Edman degradation of peptide fragment 1. 40% of the PTH-amino acid, which remained in the fraction collector after each sequencing cycle, was used for radioactivity counting. The amino acid sequence of the fragment is shown in comparison. Values are means ± SD from four different radioactivity countings of each PTH-amino acid. Indicated by asterisks are differences from background radioactivity at the level of significance: $P < 0.05$(*) and $P < 0.01$ (**).

the amount of tritium in phenylthiohydantoin amino acid derivatives was measured after each cycle. Only in peptide fragment 1 were radioactive labeled amino acids identified. Figure 8 shows the radioactivity distribution after Edman degradation of this peptide. Tritium labeling is found after cycle 2 and mainly after cycle 6. Comparison with the amino acid sequence of peptide 1 (Table II) shows that the radioactivity is incorporated in threonine and mainly in arginine. These amino acids correspond to residues 102 and 106, respectively, in the second extracellular domain of the human $V_2$ receptor homologue.

## DISCUSSION

In this work we describe the purification of the photoaffinity-labeled $V_2$ vasopressin receptor from bovine kidney. Our strategy of receptor purification is based on the covalent labeling of the membrane-bound $V_2$ receptor with a tritium-labeled photoreactive peptide ligand.

From the amount of radioactivity specifically incorporated into the $M_r$ 30 000 protein, it was estimated that the yield of the covalent reaction between the photoreactive aryl azido analogue of 1-deamino[8-lysine]vasopressin and the vasopressin receptor protein was approximately from 3 to 5%. To obtain a higher yield of the $V_2$ receptor labeling, a radioactive analogue of 1-deamino[8-lysine]vasopressin with a photoreactive benzoylbenzoyl group in position 8 and a high binding affinity ($K_D$ = 0.4 nM) has been synthesized (Eich et al., 1993). The photolabile benzoylaryl group possesses high reactivity for C–H bonds but a low reactivity toward water (Breslow, 1980; Helene, 1972); photoaffinity labeling of the substance P (Boyd et al., 1991) and cholecystokinin B receptors (Thiele & Fahrenholz, 1993) with a ligand containing this photoreactive group proceeded with a yield of 70%. However, for the bovine $V_2$ receptor the benzoylbenzoyl analogue incorporated into the $M_r$ 30 000 protein with a yield (2–3%) similar to that for the (azidophenyl)amidino analogue (Eich et al., 1993). Apparently, steric reasons may prevent a higher efficiency of labeling by the benzoylbenzoyl vasopressin analogue and result in high unspecific labeling and formation of ligand aggregates. Therefore the photoreactive analogue

Table II: Amino-Terminal Amino Acid Sequence Analysis of Labeled Truncated $V_2$ Receptor and Peptide Fragments 1–4 (Figure 7)[a]

| | amino acid sequence | | | | | | | | | | | | | | | | | amino |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| cycle | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | terminus |
| N-terminal truncated $V_2$ receptor | *Val#* | *Leu#* | **Pro** | **Gln** | **Leu** | **Ala** | **Trp** | **Asp*** | | | | | | | | | | 93 |
| peptide 1 (peak 1) | *Ala* | *Thr* | *Asp* | **Arg** | **Phe** | *Arg* | *Gly* | **Pro** | **Asp** | **Ala** | | | | | | | | 101 |
| peptide 2 (peak 2) | **Ala** | **Leu** | *Cys* | **Arg** | **Ala** | **Val** | *Lys* | *Tyr* | **Leu** | *Gln* | *Met* | **Val** | *Gly* | **Met** | **Tyr** | **Ala** | **Ser** | 110 |
| peptide 3 (peak 3) | *Gly* | *Ala* | **Arg*** | **Val** | **Ser** | *Ala* | **Ala** | *Val* | **Ala** | **Lys** | | | | | | | | 259 |
| peptide 4 (peak 4) | *Ile* | *His* | *Ala* | **Ser** | **Leu** | **Gly*** | **Pro** | **Gly** | *Pro* | *Ala** | **Glu** | **Arg** | **Ala*** | | | | | 232 |

[a] Residues identified by amino acid sequence analysis are shown in boldface type; residues in italics were deduced from the cDNA sequence of the PCR product which was cloned as described under Materials and Methods. Residues marked by # in the sequence of the N-terminal truncated $V_2$ receptor are deduced from the sequence conserved in all six cloned members of the vasopressin–oxytocin receptor family (Gorbulev et al., 1993). The numbering of the amino terminus corresponds to the deduced amino acid sequence of the cloned human $V_2$ receptor. Amino acids different in bovine and human $V_2$ receptors are marked by asterisks.

of 1-deamino[8-lysine]vasopressin with the (azidophenyl)-amidino group at $Lys^8$ was chosen for the purification of the vasopressin receptor.

The purification scheme (Table I) involved the use of anion-exchange chromatography, followed by isoelectric focusing, gel filtration, SDS polyacrylamide gel electrophoresis, and finally reversed-phase chromatography. This procedure resulted in about 100 pmol of the purified receptor protein which migrated as a single band with $M_r$ of 31 000 in SDS polyacrylamide gels.

The amount of tritium-labeled ligand incorporated in the $V_2$ receptor decreased during the extensive purification. This instability of the covalent hormone receptor complex, especially during preparative gel electrophoresis, prevented an exact determination of enrichment factors. Partial dissociation of the hormone receptor complex might be explained by the instability either of the amidino group of the reactive ligand at pH values above 7 (Ludwig & Byrne, 1962) or of covalent linkages between the activated aryl azido compound and nucleophilic groups of the receptor protein (Nielsen & Buchardt, 1982).

The molecular weight of the purified affinity-labeled vasopressin receptor is significantly less than the value of about 40 000 for cloned $V_2$ receptors calculated from their primary structures. The N-terminal sequence analysis of the purified labeled bovine vasopressin receptor shows that the purified protein with $M_r$ of 30 000 is a truncated $V_2$ receptor. The amino acid sequence surrounding the cleavage site is conserved in all cloned $V_2$ vasopressin receptors from different species and is located at the transition between the second transmembrane domain and the second extracellular region (Figure 7B). We have evidence that the $V_2$ vasopressin receptor is cleaved during the 30-min incubation with the photoreactive ligand at 30 °C but not during membrane preparation or by photoactivation of the ligand. Proteolytic processing of receptor polypeptides by endogenous proteinases has been described in other seven-transmembrane receptor systems (Boege et al., 1987; Nissenson et al., 1987 Saito et al., 1991; Kozuka et al., 1991).

To determine the receptor domains involved in hormone binding, fragmentation of the purified protein with $V_8$ protease and protein microsequencing was performed. By comparison with the sequence of a cloned PCR product, four fragments of the bovine $V_2$ receptor were identified (Table II and Figure 7B). Radioactivity after Edman degradation was found only in fragment 1 located at positions 101–110 in the second extracellular domain of the human $V_2$ receptor. The radioactivity profile of the released amino acids demonstrates (Figure 8) that the ligand is covalently bound to Arg-106 and presumably also to Thr-102. Our results suggest that these residues participate directly in hormone binding or are very close to the binding site. Residues of the second extracellular

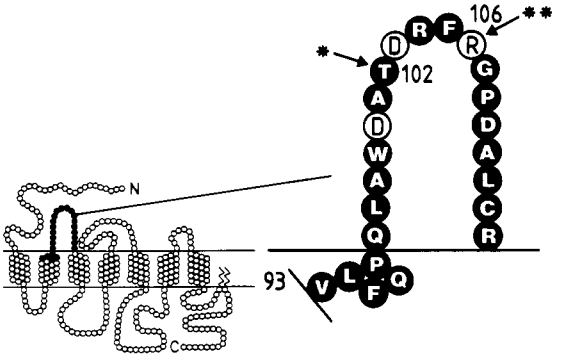


FIGURE 9: Second extracellular domain of the bovine $V_2$ receptor. Radioactively labeled amino acids identified by Edman degradation as hormone contact sites are marked with asterisks. Residues which are conserved in $V_2$ receptors are shown in white letters on a black background. Furthermore, the endogenous proteinase cleavage site between residues corresponding to $Gln^{92}$ and $Val^{93}$ in the human $V_2$ receptor is indicated.

domain may be particularly involved in binding the acyclic C-terminal tripeptide of vasopressin, where the photoreactive group is introduced into the peptide hormone. In proximity to the labeled amino acids are three aspartic residues (Asp-100, Asp-103, and Asp-109; Figure 9) which could initiate binding by ion–ion interaction with the positively charged side chain in position 8 of the photoreactive ligand or the natural hormone. The amino acid sequence of the second extracellular domain is strongly conserved between the bovine $V_2$ receptor and the $V_2$ receptors cloned from three different mammalian species (Figure 9). The labeled $Thr^{102}$ is found in all known $V_2$ receptors, the labeled $Arg^{106}$ is replaced only in the rat $V_2$ receptor by histidine. This sequence homology suggests that this $V_2$ receptor region might be involved in hormone binding in several mammalian species. Our finding that residues of the second extracellular domain are part of the hormone-binding site may be tested in future experiments by mutating these residues and assessing the effect of ligand binding.

The photoreactive ligand used for the photoaffinity labeling of the $V_2$ vasopressin receptor has agonistic properties with a high antidiuretic activity in vivo (Fahrenholz et al., 1986). Covalent binding of this ligand by photoactivation to the $V_2$ receptor on a renal epithelial cell line led to an irreversible $V_2$ receptor activation with a prolonged cAMP-mediated response (Jans et al., 1987). Our results represented here, therefore, indicate that the second extracellular segment of the $V_2$ receptor is involved in agonist binding. In proximity to this agonist binding site a proteolytic cleavage site is located in the bovine renal $V_2$ receptor. Whether a functional connection between ligand binding and truncation of the $V_2$ receptor exists has to be examined in further experiments.

We could not identify radioactive residues after protein sequencing in other receptor fragments. As the amount of peptides obtained after fragmentation was very small, our results do not exclude that other extracellular and transmembrane regions of the $V_2$ receptor are involved in ligand binding. Studies on adrenergic and muscarinic receptors have demonstrated that the binding site for these relative small molecules is located within the transmembrane helices. For ligands such as peptide hormones, especially for neurokinins, there is recent evidence from molecular genetic studies that the extracellular domains of their receptors comprise part of the ligand-binding site. For the substance P (NK 1) receptor it has been shown that all four extracellular loops might be involved in the binding of peptide agonists. With respect to our results obtained with the $V_2$ receptor it is interesting to note that two amino acid residues of the second extracellular segment are required for the optimal binding of peptide agonists by the substance P receptor (Fong et al., 1992). Analysis of chimeric endothelin A and B receptors also indicated that the second extracellular domain is involved in peptide agonist binding (Adachi et al., 1992). The results presented here demonstrate for the first time, by a direct protein chemical approach applying photoaffinity labeling, the involvement of an extracellular domain in a G-protein-coupled peptide hormone receptor for agonist binding.

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